A candidate therapeutic vaccine against hepatitis C virus infection

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A Candidate Therapeutic Vaccine Against Hepatitis C Virus Infection

Use of a Recombinant Alphavirus Vector

Peng Peng Ip

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1

General Introduction and Outline

SCOPE OF THIS THESIS

The work described in this thesis focuses on the development of a therapeutic vaccine against hepatitis C virus (HCV) infection. Approximately 150 million people worldwide are chronic carriers of HCV[1]. Currently, there is no prophylactic or therapeutic vaccine available. Several novel antiviral protease inhibitors have been approved in the last few years. These are used either alone or in combination with pegylated interferon-α and ribavirin, and have improved the cure rates considerably. However, these novel treatment modalities are very expensive, unavailable for underdeveloped countries and also sometimes associated with serious side effects. A therapeutic vaccine, which activates the immune response of patients, could be used either as a single treatment modality or be combined with antiviral drugs.

The vaccines used in this PhD study are based on an alphavirus, a recombinant alphavirus vector system derived from Semliki Forest virus (rSFV). We developed, characterized and studied the efficacy of three HCV vaccine modalities. To enhance the immunogenicity of rSFV-based vaccines, we included endoplasmic reticulum-(ER) targeting and universal helper epitopes within the vaccines. These modifications were analyzed both in the candidate HCV vaccines and in an rSFV-based therapeutic vaccine against human papillomavirus (HPV).

In this first chapter, we introduce viral- and tumor immunology and the basic concepts underlying immunotherapy. Subsequently, HCV and HPV are introduced and finally, we present an overview of the thesis.

VIRAL AND TUMOR IMMUNOLOGY

The function of the immune system is to protect against invading pathogens and derailed cells. The immune system therefore needs to recognize pathogens and distinguish pathogen-infected cells as well as cancer cells from healthy cells. Next, responses that lead to destruction of pathogens, infected cells or cancer cells are needed that do not damage healthy cells or tissues. Effective immune responses against infection and cancer thus depend on a critical balance between clearance of "foreign antigen" and maintenance of the host's integrity.

Recognition of 'foreign' by the immune system

Foreign antigens can be derived from pathogens or tumor cells. When *e.g.*, viruses enter their host, surface antigens on these viruses can already be recognized by the immune system. Viruses subsequently infect their target cells and start their reproduction cycle. Infected cells make themselves visible to immune cells by presenting pieces of degraded viral proteins (peptides) on their surface in the context of major histocompatibility complex (MHC) molecules. Peptide-MHC complexes are recognized by the T cell receptor of T cells, which represent important players of the immune system.

Although tumor cells originate from host cells that are ignored by the immune system under normal conditions, tumor cells often express tumor-associated antigens (TAAs) either as naïve proteins or as peptides complexed to MHC molecule that can be recognized by the immune system. Malignant transformation can result from heterogeneous genetic changes leading to uncontrolled cell proliferation[2]. Thus, TAAs can be derived from tissue-specific differentiation antigen, over-expressed self-antigens or mutated antigens[3-5]. And since also approximately 20% of cancers are caused by persistent infection with cancer-associated pathogens such as *Helicobacter pylori*, HPV and HCV[6], various tumors also specifically express pathogen-derived TAA.

A battle of the immune system against intruders, focusing on viruses and tumors

The immune system includes innate immunity and adaptive immunity. Innate immunity is the first line of defense against incoming pathogens. Members of the innate immunity, such as macrophages, neutrophils and natural killer cells, can quickly identify a virus by recognizing the pathogen-associated molecules on the viral surface. Although this reaction is non-specific, the fast response of the innate immunity allows an immediate attack of the invading virus[7].

Adaptive immunity, which is triggered by the innate immune system, requires recognition of the viral components and subsequently develops antigen-specific effector and memory responses. Depending on the type of response, adaptive immunity is divided into humoral and cellular immune responses. The humoral (fluid) immune response involves antibodies secreted by activated antigen-specific B cells. Antibodies recognize native antigen on the surface of the virus and lead to neutralization and clearance of circulating free viruses. Antibodies also recognize antigen on the surface of virus-infected cells or tumor cells and kill these target cells through activation of the complement system. In contrast, cellular immunity is mediated by T cells that recognize the processed antigens (peptides) presented in the context of MHC molecules on the surface of virus-infected cells or tumor cells. Activation of the adaptive cellular immune response involves (i) antigens expressed by virus-infected or tumor cells, (ii) uptake, processing and presentation of these antigens by antigen presenting cells (APCs), (iii) priming and activation of immune cells (APCs, $CD4^+$ and $CD8^+$ T cells) in lymphoid organs, (iv) trafficking of activated T cells towards the affected site, (v) infiltration of the diseased site by activated T cells, (vi) recognition of virus-infected or tumor cells by T cells and (vii) killing of target cells mainly by cytotoxic T lymphocytes (CTL). Killing of target cells leads to release of antigen resulting in further activation of the immune system (step 1 of the cycle)[8].

Viral infections can be prevented by an effective humoral immune response

as the neutralizing antibodies block the entry of virus into its target cells. However, once viruses have entered cells or tumors have developed, both humoral and cellular immune responses are required for effective killing of the affected cells. Taken together, activation of adaptive immunity should lead to eradication of virus-infected cells or tumor cells and development of antigen-specific memory response. However, this does not always happen as viruses and tumors can escape immune responses by mechanisms described below.

Regulation of the immune response

Tumor progression and persistence of viral infections requires coevolution and coexistence of the "foreign" and the host. As a consequence viruses and tumors develop strategies to reduce their immunogenicity and to allow the escape from immune surveillance. These strategies involve hiding from immune recognition and suppression of the immune system.

Viruses and tumors avoid immune recognition for example by (i) persisting in an immune-tolerant environment such as the liver, (ii) developing escape mutants and (iii) down-regulating antigen production, processing and presentation (reviewed in ref. 9-11). These mechanisms of evasion lead to non-responsiveness of the immune system or induction of adaptive immune tolerance[12]. Adaptive immune tolerance is characterized by the development of anergic T cells (both $CD4^+$ and $CD8^+$ T cells) that are not able to attack virus-infected and tumor cells[13].

Virus-infected and tumor cells suppress the function of T cells and other immune cells *e.g.*, APCs and nature killer cells by (i) expressing inhibitory molecules such as programmed death receptor ligand-1 (PD-L1), (ii) secretion of immunosuppressive molecules such as transforming growth factor (TGF)-β and indoleamine 2,3-dioxygenase (IDO), or (iii) by recruitment of suppressive immune cells such as regulatory T cells, myeloid derived suppressive cells (MDSCs) and tumor-promoting tumor-associated macrophages to the affected area[14,15]. Thus, suppression of immune system may result in persistence of viruses and tumors[15-17].

As escape immune surveillance is observed in both viral diseases and cancer, activating the immune system could possibly support the combat against these diseases.

TREATMENT AGAINST CHRONIC VIRAL INFECTIONS AND TUMORS

Current standard treatment modalities against virus infections and cancer in general directly target viruses, virus-infected cells or tumor cells. And although some of these standard treatments may indirectly or as a consequence of cell death also augment immune responses, antiviral and cytostatic drugs are not primarily designed for this. Yet, with our emerging knowledge and activation methods of immune responses,

immunotherapy will likely in the near future develop as a novel treatment modality for infections and cancers. In fact, improvement in cancer immunotherapy was selected by *Science* as the "breakthrough of the year 2013"[18].

Immunotherapeutic strategies

Immunotherapy includes treatments that induce or boost specific immune responses. To boost existing immune responses, immune modulators so-called check-point blocking antibodies such as anti-cytotoxic T lymphocyte antigen (CTLA)-4 and anti-PD-L1 antibodies are being used to activate T cells and restore the function of resident anergic/exhausted T cells, respectively[19,20]. These promising immune modulators lead to prolonged survival of cancer patients[21]. Ways to induce and/or boost immune responses involves for example adoptive transfer of specific T cells[22] or APCs and delivery of therapeutic vaccines.

Therapeutic vaccines

Induction of immune response can be achieved with different vaccine platforms that are based on proteins, synthetic peptides, DNA plasmids, virus-like particles or bacterial and viral vectors. Each platform has its pros and cons with respect to production, costs, efficacy and safety[23]. We focused on the Semliki Forest virus (SFV) system to deliver our antigen of interest. Viral vectors generally induce robust innate and adaptive immune responses and therefore are potent tools for therapeutic vaccine development. The most commonly used viral vector vaccines are based on adenoviruses or vaccinia viruses. Both vector systems can induce strong immune responses. However, pre-existing vector-specific neutralizing antibodies in patients developed by natural infection and/or repeated vaccination, may hamper the efficacy of these vaccines[24]. Vaccines based on Alphaviruses, such as Venezuelan Equine Encephalitis virus (VEE), Sindbis virus (SIN) or SFV, are highly promising. Pre-existing immunity against these viruses is rare in human. Moreover, it has been shown in mice that pre-existing antibodies against SFV do not hamper immune responses[25].

The recombinant Semliki Forest virus (rSFV) system

Semliki Forest virus belongs to the Alphavirus genus of the *Togaviridae* family[26]. The *Togaviridae* are a family of viruses with a linear, single-stranded, positive sense RNA genome. The SFV vector system is based on the self-replicating ("replicon") properties of the positive-strand RNA genome of SFV. In rSFV replicon particles, the recombinant RNA genome encodes the viral RNA replicase and the gene(s) for the antigen(s) of interest, but lacks the genes for the structural proteins of the virus. Thus, in a single round of infection, the virus penetrates targets cells producing large amounts of the antigen(s) involved without generating new progeny virions. The rSFV replicon particles are thus replication-incompetent[27]. rSFV replicon particles

can infect a wide variety of cells, activate both innate and adaptive immunity[28-31] and induce high expression of the antigen of interest[32,33]. Within 48-72 hours after infection, rSFV-infected cells undergo programmed cell death[34,35]. This results in release of a large amount of antigen and the subsequent initiation of a robust adaptive immune response.

In preclinical studies, rSFV-based vaccines have been shown to induce strong and protective immune responses against foreign proteins such as antigens from viruses, bacteria and tumors[36]. Because of the high level of transgene expression, rSFV can also be used for temporary delivery of immune modulators such as anti-tumoral cytokines, IL-12 (ref. 37). There is no clinical study on rSFV yet. However, recombinant VEE-based vaccines, which are constructed in a similar way as rSFV, are now being studied in phase I/II clinical trails. These include prophylactic vaccines against human immunodeficiency virus (HIV), influenza A virus and cytomegalovirus (CMV) and a cancer vaccine against colorectal cancer and breast cancer[38].

TUMOR-ASSOCIATED VIRUSES

As described earlier, some tumors arise as a consequence of infection with a virus. In 1910, Peyton Rous described that a spindle cell sarcoma of a hen is transmissible with tumor filtrates[39]. The virus in the tumor filtrates was identified and named as Rous sarcoma virus and it was the first identified tumor-associated virus in mammals[40]. Nowadays, six human viruses are recognized as tumor-associated viruses and contribute to 10-15% of global cancer[6]. Tumor formation is caused by inflammation due to chronic infection with hepatitis B virus, HCV or by persistent expression of oncoproteins by Epstein-Barr virus, HPV, human T-cell lymphotropic virus and Kaposi's associated sarcoma herpesvirus (KSHV)[41]. Since this thesis includes studies of rSFV-based HCV and HPV vaccines, a general introduction of these viruses is given below.

Hepatitis C virus

Hepatitis C virus (HCV) is a small, enveloped, positive-sense single-stranded RNA virus belonging to the Hepacivirus genus of the family *Flaviviridae*. The viral RNA genome (9.6 kb) is consists of a 5' and 3' UTR and one open reading frame encoding a viral polyprotein. This polyprotein is post-translationally cleaved into three structural proteins (core, envelope 1 and 2), a small membrane polypeptide p7 and six nonstructural proteins (nsPs) (NS2, NS3, NS4A, NS4B, NS5A and NS5B). The function of these proteins is described in detail by Lindenbach and Rice[42]. Until now, 7 genotypes and more than 50 subtypes of HCV have been identified. HCV quasispecies are escape mutants evolved in patients as a result of the high mutation rate as well as the host's immune pressure[43,44]. HCV is transmitted by blood and mainly

infects hepatocytes. Spontaneous resolution occurs in 10 to 15% of HCV-infected patients who do not receive any treatment. The majority of HCV-infected patients acquire a persistent infection correlating with the development of liver cirrhosis, liver failure and hepatocellular carcinoma. Current treatment includes a combination of pan-antiviral drugs and HCV-specific protease inhibitors (direct acting antivirals, DAAs) that primarily target the replication of the virus[45].

A broad and robust T-cell response against HCV nsPs is key to resolve acute HCV infection[17,46-48]. Viral-neutralizing antibodies have been identified in patients[49,50]. However, the role of humoral immunity against HCV is still controversial, as antibody-deficient patients have been shown to recover from acute HCV infection[51]. HCV evades the host immune response through direct or indirect action of viral proteins[52-54]. Moreover, patients with a chronic HCV infection have reduced diversity of HCV-specific T cell with exhausted phenotypes[55,56]. Therefore, besides antiviral treatment, induction and augmentation of T-cell responses against HCV antigen is required for effective clearance of the infection.

Various therapeutic approaches using protein, plasmid DNA and viral vectors are being developed. Viral vectors so far induce the most robust cellular immune response in preclinical studies[57]. For example, it has been shown that rSFV expressing the NS3 and NS4A proteins of HCV is able to induce robust NS3-specific responses in mice[58]. In order to broaden the spectrum of the T-cell response, we generated rSFV expressing all HCV nsPs and studied its vaccine potency (**Chapter 3**).

Human papillomavirus

Human papillomavirus (HPV) is a small non-enveloped double-stranded DNA virus (8 kb) belonging to the Papillomavirus genus of the family *Papovaviridae*. The DNA genome consists of six early (E1, E2, E4, E5, E6 and E7) and two late genes (L1 and L5)[59]. Over 190 HPV genotypes have been identified of which 40 genotypes are classified as sexually transmissible HPV[60]. Fifteen HPV types are considered to have oncogenic potential and are therefore classified as high-risk HPV types[61]. The high-risk HPV types 16 and 18 are associated with 54.6% and 15.8%, respectively, of cervical cancer[62]. Treatment depends on the stage of disease, generally involving surgery and/or radiotherapy and chemotherapy. Combined treatment achieves a curative rate of approximately 90%, however, it may also come with side effects such as infertility[63].

HPV infection initially occurs at the basal layer of the cervical squamous epithelia that is exposed by micro-abrasions. Although the majority of people infected with a high-risk HPV type clear the infection, persistent infections may lead to (pre)malignancy. Persistent/latent HPV infection is characterized by the presence of episomal HPV DNA in the nucleus of infected epithelial cells followed by the integration of HPV DNA genome into the chromosome of the infected epithelial

cells. This results in uncontrolled expression of HPV viral proteins[64]. Development of HPV-associated lesions is mainly attributed to the expression of HPV oncogenic early proteins E6 and E7 (ref. 65). Furthermore, HPV is capable to escape from the host immune surveillance enabling persistent infection and cervical cancer progression[66].

Immunotherapy against HPV aims to induce cellular responses against cells expressing the oncogenic proteins E6 and E7 (ref. 67-69). For most vaccine strategies such as peptide and protein vaccinations, a robust cellular response is achieved by inclusion of immune modulators that act as an adjuvant to boost the overall immune response[70]. For DNA vaccines, carrier proteins are extensively used in order to directly or indirectly augment the HPV-specific cellular responses[71]. Our group demonstrated that rSFV expressing HPV E6 and E7 induced protective cellular immune response that outperformed other vaccination strategies such as protein, DNA and adenoviral vectors[72,73]. Since carrier proteins used in DNA vaccines highly up-regulate the vaccine immunogenicity, we investigated the possible roles of immunogenic carrier proteins in rSFV vectors (**Chapter 4, 5**).

OUTLINE OF THIS THESIS

Chapter 2 reviews literature on HCV therapeutic vaccines (until 2012) in both preclinical and clinical studies.

Chapter 3 describes the production and characterization of rSFV replicons particles expressing all or a part of the nsPs of HCV in order to induce a robust and broad-spectrum immune response. Inclusion of all HCV nsPs induced a strong and long-lasting HCV-specific $CD8^+$ T-cell response in mice. Furthermore, immunization with rSFV expressing all nsPs of HCV significantly reduced the outgrowth of HCV-expressing tumors.

Chapter 4 describes strategies to augment rSFV vaccine immunogenicity with inclusion of various immunogenic carrier proteins. Since carrier proteins used in DNA vaccines highly up-regulate the vaccine immunogenicity, we investigated the possible roles of immunogenic carrier proteins in the rSFV system. We generated, characterized and evaluated the efficacy of rSFV vectors expressing HPV E6 and/or E7 proteins fused to a protein expressing helper T cell (Th) epitopes, tetanus toxin fragment C (TTFC) or a series of Th epitopes and an endoplasmic reticulum targeting signal (sigHELP-KDEL). Inclusion of the sigHELP-KDEL strongly increased the frequency of E7-specific T cell and protected mice from tumor formation even with very low immunization doses of rSFV.

Chapter 5 presents a preliminary study on the effect of the inclusion of the immunogenic carrier protein described in Chapter 4, sig-HELP-KDEL, into

the HCV-nsPs-expressing rSFV replicon. Immunization with sigHELP-KDEL-expressing rSFVeNS3/4A (rSFVe-sHELP-NS3/4A) induced similar high levels of functional NS3-specific T cells as compared to immunization with rSFVeNS3/4A. Further investigations have to be performed to clarify the effect of the inclusion of sigHELP-KDEL on HCV-expressing tumor growth.

Chapter 6 describes an *in silico* approach to identify HCV T cell epitopes which can be used for the rational design of a tailor-made therapeutic HCV vaccine. An *in silico* approach which includes both the prediction of CTL and Th epitopes as well as proteasomal cleavage sites with multiple mathematic algorithms is presented. Furthermore, the binding affinity and immunogenicity of the identified CTL epitopes were validated *in vitro* and *in vivo*.

Chapter 7 presents a summarizing discussion of the studies in this thesis and a future perspective on the development and application of a therapeutic HCV vaccine.

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Therapeutic Vaccination Against Chronic Hepatitis C Virus Infection

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Therapeutic Vaccination Against Chronic Hepatitis C Virus Infection

ABSTRACT

Approximately 170 million people worldwide are chronic carriers of Hepatitis C virus (HCV). To date, there is no prophylactic vaccine available against HCV. The standard-of-care therapy for HCV infection involves a combination of pegylated interferon-α and ribavirin. This therapy, which is commonly associated with side effects, has a curative rate varying from 43% (HCV genotype 1) to 80% (HCV genotype 2). In 2011, two direct-acting antiviral agents, telaprevir and boceprevir, were approved by the US Food and drug Administration and are now being used in combination with standard-of-care therapy in selected patients infected with HCV genotype 1. Although both drugs are promising, resulting in a shortening of therapy, these drugs also induce additional side effects and have reduced efficacy in patients who did not respond to standard-of-care previously.

An alternative approach would be to treat HCV by stimulating the immune system with a therapeutic vaccine ideally aimed at (i) the eradication of HCV-infected cells and (ii) neutralization of infectious HCV particles. The challenge is to develop therapeutic vaccination strategies that are either at least as effective as antiviral drugs but with lower side effects, or vaccines that, when combined with antiviral drugs, can circumvent long-term use of these drugs thereby reducing their side effects.

In this review, we summarize and discuss recent preclinical developments in the area of therapeutic vaccination against chronic HCV infection. Although neutralizing antibodies have been described to exert protective immunity, clinical studies on the induction of neutralizing antibodies in therapeutic settings are limited. Therefore, we will primarily discuss therapeutic vaccines which aim to induce effective cellular immune response against HCV.

1. INTRODUCTION

The World Health Organization (WHO) has estimated that about 3% of the world population is infected with hepatitis C virus (HCV) and approximately 170 million individuals are chronic carriers of HCV. In this group of chronic carriers, 5-10% are at risk of developing liver cirrhosis and liver cancer[1]. Each year, 3-4 million people are newly infected with HCV and 350,000 patients die from HCV-related disease. HCV infection is distributed worldwide (**Figure 1**) with high rates of chronic infection in Egypt (22%)[2], Pakistan (4.8%)[3] and China (3.2%)[4]. The standard-of-care treatment for patients infected with HCV is a combination of pegylated interferon-α and ribavirin. This treatment is generally associated with side effects and is effective in 43% and 80% of the patients with genotype 1 and genotype 2 infections, respectively[5-7]. A prophylactic or therapeutic vaccine against HCV is not yet available. Yet, it is known that immune responses against HCV do play a significant role in viral clearance. While patients recovering from an acute HCV infection mount an effective cellular and humoral immune response against HCV; in individuals with a chronic infection, T-cell responses are often deficient and production of neutralizing antibodies is delayed.

An ideal therapeutic vaccine to treat patients with a chronic infection aims at the induction of both cellular and humoral immune responses. HCV-specific cellular immune response to effectively clear HCV infected cells and humoral response to reduce the amount of circulating HCV particles. And, humoral immune responses against HCV have been shown to also play a role in clearance of infected cells by antibody-dependent cellular cytotoxicity *in vitro*[8].

Several approaches are currently being studied to develop vaccines that induce or reactivate a robust T-cell response. Yet, so far studies on the induction of neutralizing antibodies in therapeutic settings are limited. In this review, we will therefore summarize different preclinical vaccine candidates, vaccine formulations, approaches and clinical studies which aim to induce HCV-specific cellular immune responses in patients with a chronic HCV infection (see **Table 1**). However before reviewing these vaccination strategies we will, in **Section 2**, briefly describe the virology and pathology of HCV and the standard-of-care treatment for HCV patients. Next, in **Section 3**, we will address immune mechanisms in acute HCV-infected patients that either result in clearance of the virus or result in chronic disease. In **Section 3** we will also address the problems and challenges associated with the development of prophylactic and therapeutic HCV vaccines.

2. HEPATITIS C: VIROLOGY, PATHOGENESIS AND CURRENT THERAPY

2.1. Virology

HCV was first identified by Houghton and colleagues as non-A, non-B hepatitis virus

Table 1. Recent clinical studies relating to therapeutic vaccination against HCV infection.

Delivery system	Commercial name	HCV antigen	HCV genotype	HLA restriction	Study phase	Adjuvant	Participants*	Clinical outcome	References or ClinicalTrials.gov Identifier
Peptides	IC41 (Intercell AG, Vienna; Verena)	core 23–44; core132–140; NS3 1073–1081; NS3 1248– 1261; NS4 1764–1786	1	HLA-A*0201	Phase I	+ Poly-$_L$-arginine	c, d	Induction of HCV-specific central memory as well as effector $CD8^+$ T cells in healthy subjects. Increase of HCV-specific $CD8^+$ T cells with a decline of $CD45RA^+$ effector memory cells in some but not all patients.	94
					Phase II	+/- Poly-$_L$-arginine	c, d	Increase of $CD4^+$ T cell proliferation (67%); Increase of IFN-γ ELIspot (42%); >1 log declines of HCV serum RNA (3 of 60 patients)	93
					Phase I	+ Poly-$_L$-arginine and +/- Imiquimod	a	Increase of $CD4^+$ T cell proliferation (60%); Increase of tetramer-binding and IFN-γ $CD8^+$ ELIspot (70%)	102
	None	E1 213-221; E2 488-496; NS3 1081-1090; NS5A 2132-2140	1b	$HLA-A24^+$	Phase I	Montanide ISA 51 VG	c	Increase of peptide-specific CTL activity and IgG. Decrease of HCV serum level (3 of 12 patients)	95
	None	C 35-44 (HLA-A2-restricted CTL epitope)	1a, 1b, 2a, 3a	HLA-A2, HLA-A24, HLA-A26, HLA-A31, HLA-A33	Phase I	Montanide ISA 51 VG	b, c	Increase of peptide-specific CTL activity (15 of 25 patients), Increase of peptide-specific IgG (15 of 22 patients). >1 log declines of HCV serum RNA (2 of 25 patients)	97
DNA	CIGB-230	pIDKE2 expressing Core, E1 and E2 protein and recombinant HCV core proteins, Co120.	1b, Cuban isolated	None	Phase I	None	c	Increase of specific T cell proliferative response and T cell IFN-γ ELIspot (73%). Improved or stabilized liver histology (>40%).	123
		CIGB-230 and recombinant anti-Hepatitis B vaccine (Heberbiovac HB, Cuba)	1b, Cuban isolated	None	Phase I	None	c	Induction of specific $CD4^+$ T cells response (42.8%).	124
	ChronVac-C® + standard-of-care	NS3/4A	1	None	Phase I and II	None	b	Phase I: sero-conversion of HCV RNA (5 of 6 patients)	NCT01335711
Viral vector	TG4040	MVA virus carrying NS3, NS4 and NS5B	1	None	Phase I	None	b, d	Decline in serum HCV RNA (6 of 15 patients)	149
	HCVac (TG4040 + standard-of-care)	MVA virus carrying NS3, NS4 and NS5B	1	None	Phase II	None	b	Ongoing	NCT01055821
	None	Ad6 or Chimpanzees adenoviral (ChAd3) vector carrying complete NS proteins	1b	None	Phase I	None	a	Induction of long-lived $CD4^+$ and $CD8^+$ T cells response with central and effector memory phenotypes.	132
Yeast	GI-5005	Core-NS3 fusion protein	1a and 1b	None	Phase Ib	None	b, c, d	Decline in serum HCV RNA	NCT00124215
	GI-5005 + standard-of-care	Core-NS3 fusion protein	1a and 1b	None	Phase II	None	b, c	Ongoing	NCT00606086
Dendritic cells	None	HCV CTL epitopes: core 132-140; core 35-44; core 177-187; NS3 1406-1415; NS4B 1807-1816; NS4B 1851-1859; universal Th epitopes	1	HLA-A2	Phase I	Pam_2Cys	c	All patients generated *de novo* specific-IFN-γ response.	180

* a. Healthy subjects; b. HCV-infected patients without (or refused) the standard-of-care therapy; c. HCV-infected patients not responding to the standard-of-care therapy; d. HCV-infected patients relapsing from the standard-of-care therapy

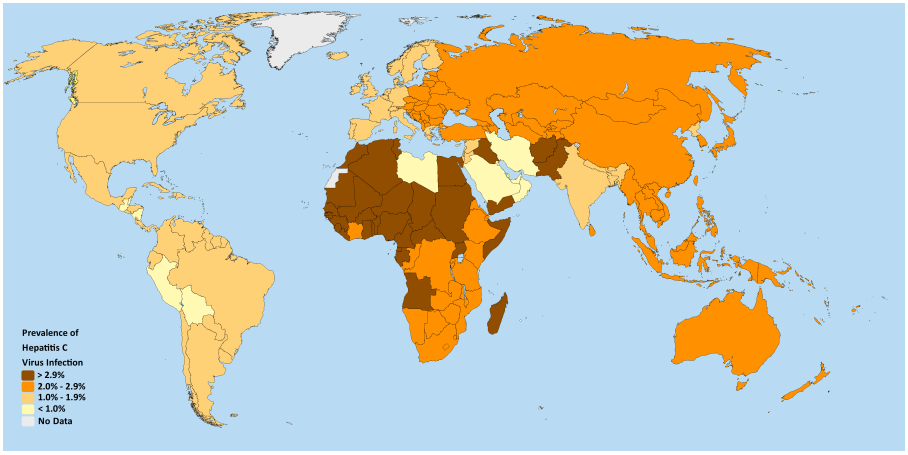

Figure 1. Prevalence of hepatitis C virus infection. *Sources*: The US Centers for Disease Control and Prevention, Hepatitis C virus database project.

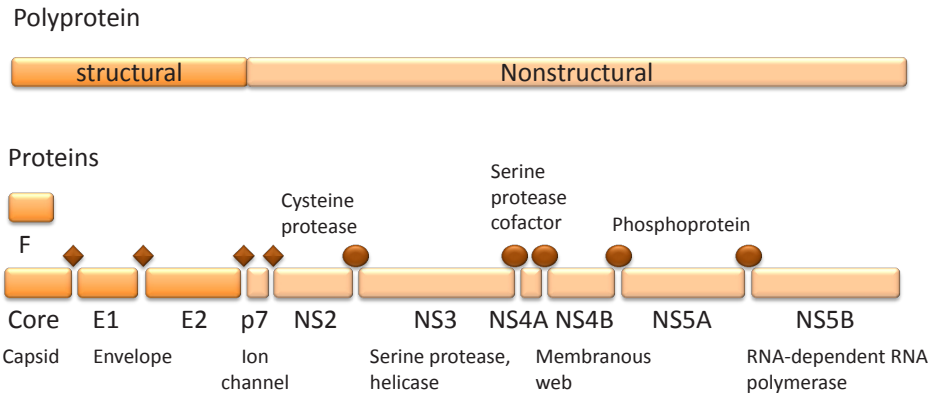

Figure 2. Hepatitis C virus proteins. The HCV polyprotein is processed by cellular proteases (diamonds) or viral proteases (circles) into 10 viral proteins. The basic function of each protein is indicated and extensively discussed in reference 19.

in 1989 (ref. 9). HCV is a small enveloped, positive-sense single-stranded RNA virus belonging to the family *Flaviviridae* in the genus Hepacivirus. The 9.6 kb viral RNA genome is composed of one open reading frame encoding a polyprotein of 3000 amino acid residues, and a 5' and 3' UTR. HCV enters cells through receptor-mediated endocytosis. Several cellular receptor proteins for HCV have been identified. Among them are tight-junction proteins occludin and claudin-1, scavenger receptor class B type I, and the tetraspanin CD81 (ref. 10-13). Since HCV particles are associated with lipoproteins such as LDL and VLDL[14,15], binding of viral particles may be initiated by interaction of the lipoprotein on the viral particles and their corresponding receptors expressed on host cells[16], followed by recognition through other cellular receptors. Recently, Sainz and coworkers demonstrated that, besides the scavenger receptor B type I and the LDL receptor, also the Niemann-Pick C1-like cholesterol adsorption

receptor is involved in HCV entry[17]. Upon entry into the cytoplasm, the viral RNA genome is translated to a polyprotein that is post-translationally cleaved by viral and cellular proteases into three major structural viral proteins, a small membrane polypeptide p7 and six non-structural proteins (nsPs). The structural proteins are core (C), envelope (E) 1 and 2, and the nsPs are NS2, NS3, NS4A, NS4B, NS5A and NS5B (**Figure 2**). HCV encodes a small protein, F (or ARFP, for "alternative reading frame protein"), which is produced by a ribosomal frame shift within the core gene[18]. The function of each of the viral proteins is discussed in ref.19.

To date, at least seven genotypes and 50 subtypes of HCV have been isolated worldwide and the presence of HCV quasispecies can be detected in HCV-infected individuals. HCV has a high production rate of 10^{12} particles per day with a half-life of 2.5 hours. With the high error rate of the viral RNA-dependent RNA polymerase, the mutation rate of the HCV genome has been estimated to be 1.92×10^{-3} base substitutions per site per year[20]. This mutation rate is higher than that of human immunodeficiency virus (HIV) type 1 which is 3.4×10^{-5} base substitutions per site per year[21]. These frequent mutations result in production of viral variants which may not only improve replication fitness but also hampers the host immune system to recognize and clear the virus[22].

2.2. Pathogenesis

HCV represents a major cause of liver disease in humans and chronic HCV infection is also the primary cause for liver transplantation in the Western world. HCV is transmitted mainly by blood transfusion, re-use of medical devices and shared needles among injection-drug users. Both innate and adaptive immune responses are required for viral clearance and will be discussed in detail below. Inefficient immune responses and chronic HCV infection induce liver damage and inflammation which may lead to liver cirrhosis, liver failure and ultimately hepatocellular carcinoma. Hepatocarcinogenesis is caused by the interaction of several indirect mechanisms including chronic inflammation, steatosis, fibrosis and oxidative stress[23].

2.3. Standard-of-care therapy and novel drugs

The standard-of-care therapy of patients with chronic HCV infection consists of weekly injections of pegylated interferon-α and a twice-daily intake of ribavirin for 24-48 weeks. A sustained virological response defined as the absence of HCV RNA in serum for 6 months or longer after therapy, is achieved in about half of the treated patients. This treatment in most patients leads to side effects such as influenza-like symptoms and depression and about 20% of treated patients develop anemia[7]. Two new direct-acting antiviral (DAAs) drugs, INCIVEK™/ INCIVO® (telaprevir) and VICTRELIS™ (boceprevir), which both specifically inhibit the activity of the HCV NS3/4A protease, were licensed in 2011 by the US Food and Drug Administration

to be used in combination with the standard-of-care therapy for patients infected with HCV genotype 1 (ref. 24, 25). New treatment schedule with either telaprevir or boceprevir is promising. In treatment-naïve populations response-guided therapy can be shortened to 24 weeks in approx. 55% of patients treated with the telaprevir-based regime and to 28 weeks in approx. 45% of patients treated with the boceprevir-based regime[26]. Side effects such as rash and anemia occur in around 40% of the treated patients[24,25]. In two large phase 3 studies, the SPRINT-2 study (boceprevir in combination with pegylated interferon-α and ribavirin) and the HCV RESPOND-2 study (Retreatment with HCV Serine Protease Inhibitor boceprevir and PegIntron/ Rebetol 2), more than 40% of patients developed anemia and required erythropoietin administration for up to 150 days[25,27]. Presently, many other drugs inhibiting HCV viral proteins or host proteins essential for viral replication such as cyclophilin A inhibitors, including alisporivir, are under investigation. However, due to the error-prone replication of HCV, combinations of antiviral drugs as a cocktail treatment will be required to prevent development of drug resistance. On the other hand, combination therapy may lead to side effects induced by drug-drug interaction of these new DAAs. Alternatively, therapeutic vaccines may, in combination with DAAs, not only reduce the duration of the standard-of-care therapy, but may also cure patients that do not respond to the standard-of-care therapy.

3. IMMUNE RESPONSE AND VACCINATION AGAINST HCV INFECTION

3.1. Immune responses in acute HCV-infected patients

Based on the level of viral load and alanine aminotransferase (ALT) in serum, HCV infection is divided into three stages, which are the incubation, the acute and the chronic phase. During the incubation phase, HCV is actively proliferating in the liver with a high level of HCV RNA detected in serum. However, this period is mostly asymptomatic and therefore difficult to be diagnosed. Upon infection, the viral dsRNA replication intermediates of the HCV RNA activate host innate immunity through Toll-like receptor 3 (TLR3) and the polyuridine motif at 3' UTR activates the retinoic acid-inducible gene I (RIG-I) signaling in a viral 5' triphosphate-dependent manner[28]. This results in production of IFN-β by infected hepatocytes which is a first line of defense to control further infection by inducing antiviral IFN-α production in neighboring uninfected hepatocytes as described by Rehermann[29]. Furthermore, HCV NS3/4A protein also reduces the production of IFN-α by infected hepatocytes by inhibiting TLR3 and RIG-I signaling through the cleavage of the adapter molecule TRIF (Toll-IL1 receptor domain-containing adaptor inducing IFN-β) and IPS1 (IFN-β promoter stimulator protein 1), respectively[30,31]. The incubation phase with the activation of innate response lasts for 8-12 weeks and HCV infection can be diagnosed only when the levels of ALT and HCV-specific T cells increase which

characterize the acute phase of the disease[29].

During the acute phase of the disease, the level of HCV RNA fluctuates coincidentally with the level of ALT. HCV-specific T cells and HCV-specific antibodies are detectable at 5-9 and 8-20 weeks after primary HCV infection, respectively[32-34]. HCV-specific adaptive immune responses are mild and delayed, which may be due to poor antigen presentation in the immune-tolerant environment of the liver[35] and to direct inhibitory effects of HCV proteins. A protective role of anti-HCV antibodies is still controversial as it has been shown that antibody-deficient patients can recover from acute infection of HCV in the absence of anti-HCV antibodies[36]. Other studies demonstrate that neutralizing antibodies, induced in the early phase of disease, positively correlate with control of virus replication[37] and resolution[38], reducing the chance to develop a chronic infection. Because of lack of an appropriate animal model for HCV infection, the potency of neutralizing antibodies was studied in a human liver-chimeric mouse model (Alb-uPA/SCID). These mice lack an adaptive immune system and are susceptible to HCV infection. Alb-uPA/SCID mice injected with neutralizing antibodies derived from patients with chronic HCV infection were protected from homologous HCV[39,40] or heterologous HCV challenges[41].

Cellular immune responses have been shown to play a crucial role in clearance of HCV as proliferation of HCV-specific T cells and increased levels of IFN-γ coincide with the first decline of HCV RNA level in blood. Virus clearance depends on the diversity and effector function of pathogen-specific $CD8^+$ T cells. HCV-specific $CD8^+$ T cells isolated from patients at early onset of the disease (1 week) exhibit a broad antigen specificity. Yet these cells often seem to have impaired effector functions, including proliferative capacity, IFN-γ production and cytotoxicity[42,43]. The effector functions of HCV-specific $CD8^+$ T cells in patients who later recover from the acute disease improve over time. In patients who develop a chronic HCV disease this change does not occur[43]. HCV-specific $CD4^+$ T cells, perform differently between patients. Robust proliferation of HCV-specific $CD4^+$ T cells is detectable in patients who later recover from acute disease, but not in patients who progress to chronic infection. Only a minority of patients recovers from acute infection without treatment. However, these patients in general develop protective immunity against re-infection[44-46]. Protective immunity against re-infection, which has been shown to be T cell-dependent[47], is characterized by a reduced duration of viremia and a lower amount of viral RNA in the serum of homologous or heterologous HCV re-infected patients[46].

3.2. Failure of immune responses in chronic HCV-infected patients

A majority of primary HCV-infected patients (60-80% of those infected with HCV genotype 1) develop chronic infection characterized by stable HCV serum RNA levels for decades. These HCV RNA levels are approximately 2-3 logs lower than the levels

during the acute phase. The induction of virus-neutralizing antibodies is delayed but detectable during the entire period of chronic infection. Most importantly, HCV-specific $CD8^+$ T cells isolated from chronic patients are functionally exhausted which ultimately leads to a reduced breadth of the response[48,49]. T cell exhaustion in general and also in chronic HCV infection is caused by a number of factors including:

- long-term antigen stimulation in the presence of a persisting substantial (HCV) viral load;
- upregulation of anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF-β) and downregulation of pro-inflammatory cytokines such as IFN-γ, IL-2 and IL-21;
- reduced help from antigen-specific $CD4^+$ T cells;
- expression of inhibitory receptors including programmed cell death 1 (PD1), lymphocyte activation gene-3 (LAG3), CD244 (2B4), CD160, T cell immunoglobulin and mucin domain-containing molecule (TIM3) and CTL4 on the surface of antigen-specific T cells[50];
- presence of immune suppressor cell populations such as regulatory T cells (Treg)[51].

For instance, several groups reported on the presence of high frequencies of both Treg and HCV-specific $CD8^+$ T cells expressing PD1, CTLA4 and TIM3 in the periphery and liver of patients with chronic HCV infection[52-54]. Exhaustion occurs for both $CD4^+$ and $CD8^+$ T cells. Exhaustion of $CD8^+$ T cells is characterized by a gradual progression of dysfunction. It begins with a reduced, and later a complete lack of, production of tumor necrosis factor α (TNF-α) and IL-2, followed by a reduced production of IFN-γ and loss of cytolytic function. At later time points, expression of inhibitory receptors such as PD1 and TIM3 is induced and apoptosis begins[51]. Exhaustion of $CD4^+$ T cells is not fully understood but it has been shown that HCV-specific $CD4^+$ T cells isolated from patients with chronic disease have a reduced production of IL-2 and IFN-γ[55]. Since dysfunction of HCV-specific $CD4^+$ T cells is not observed in patients who clear the infection, this may provide clues as to how to treat patients with a chronic infection. If the function of exhausted HCV-specific T cells can be restored and the diversity of HCV-specific T cells will be broadened, patients with a chronic infection may regain the ability to clear the virus. Fortunately, exhaustion of T cells is reversible; for example, it has been shown that HCV-specific cytotoxic T lymphocyte (CTL) activity can be restored by blockade of either PD1/CTLA4 or TIM3 (ref. 56, 57). The function of exhausted T cells can also be restored by elimination of the original causes of dysfunction. For example, such as to increase help from $CD4^+$ T cells, to provide adequate amount of IFN-γ, IL-2 and IL-21 and to inhibit the function of Treg. All these strategies should be taken into account in the design of therapeutic vaccines against HCV infection in order to restore exhausted T cells and to increase the diversity of HCV-specific T cells.

Viral mutants or quasispecies, appearing under immune pressure[58] may not be recognized by an intact immune system despite the fact that patients develop a spectrum of functionally active HCV-specific T cells[22]. Mutations can be found in various regions of the viral genome including T cell epitopes, B cell epitopes or other regions. For example, viral mutants which have mutations within neutralizing epitopes have been described to infect hepatocytes more efficiently than their consensus virus in the transplanted liver of patients with chronic HCV infection[59]. Mutations within T cell epitopes are positively correlated to the development of chronic disease in both chimpanzees[60] and humans[61]. Recent studies show mutations in protective HLA-B27-restricted epitopes in patients who developed chronic disease but not in those who recovered from acute infection[62,63]. Of note, the rate of mutation in CD8 T cell epitopes is comparable to the hypervariable region 1 (HVR-1) of the E2 gylcoprotein, the most variable region in the HCV genome[22]. The lack of immune recognition of these mutants was demonstrated in studies which showed that HCV-specific T cells isolated from patients can be stimulated by consensus but not by mutated CD8 T cells epitopes[22,64]. Immune escape mutations may occur on the costs of viral fitness as the antiviral effect of T cells forces the virus to adopt a relatively unfavorable sequence[58,65]. In fact, half of the detectable CD8 T-cell responses observed in patients with chronic disease were found associated with viral mutations[64]. Chronic HCV infection therefore results from the selection of immune escape mutants and the existence of functionally exhausted T cells.

3.3. Vaccination against HCV

3.3.1. Prophylactic vaccination

Conventional prophylactic vaccines against viral infections generally aim at induction of a humoral immune response resulting in the production of virus-neutralizing antibodies that eventually block receptor binding and cell entry of the viral pathogen involved. Although the neutralizing function of antibodies against the HCV envelope glycoproteins E1 and E2 in humans is still controversial[66,67], vaccination strategies which induce production of neutralizing antibodies against E1 and E2 do protect HCV-naïve chimpanzees from virus challenge[68,69]. Several studies have now shown that polyclonal antibodies against HCV may prevent the attachment to and entry of virions into hepatocytes[70-74]. Although E1 gylcoprotein-specific antibodies can be identified in patients with chronic HCV infection[74], the major antigenic determinants on the viral surface are located in the HVR-1 of the E2 glycoprotein[75] and this variability occurs not only between patients but also within a single patient making the design of prophylactic vaccines difficult. One possible solution is to delete the HVR-1 region from E2 glycoprotein allowing the presentation of other epitopes that may induce protective virus-neutralizing antibody responses. A recent study by Verstrepen and colleagues shows that virus-neutralizing antibodies against E1

glycoprotein can indeed clear HCV in chimpanzees in a T-cell-dependent manner[76]. Furthermore, a study in macaques has shown that antibodies against E1 and E2 glycoproteins possess neutralizing ability against both homologous and heterologous virus[77]. Clarification of the immunogenicity of E1 and E2 glycoproteins and the protective effect of neutralizing antibodies is essential for a more precise formulation of prophylactic and therapeutic HCV vaccines. To date, more neutralizing epitopes have been identified in patients, which may facilitate the development of prophylactic vaccines. Recently, in a phase I clinical study in healthy humans, E1- and E2-specific antibodies with *in vitro* virus-neutralizing activity were induced by vaccination with recombinant E1 and E2 protein and MF59 adjuvant[78].

3.3.2. Therapeutic vaccination

Therapeutic vaccines preferably activate both humoral and cellular immune responses, thereby generating antibodies and virus-specific $CD8^+$ cytotoxic T lymphocytes (CTL) that neutralize the circulating virus and eliminate virus-infected cells, respectively. The majority of the viral epitopes recognized by CTLs and $CD4^+$ T cells in patients with HCV infection are located in the NS3 region[32,79-86]. Therefore, NS3 is considered to be a good cellular target candidate for a therapeutic vaccine. Yet as also CTL and $CD4^+$ T cells recognizing epitopes from other conserved HCV proteins such as core and NS5A/B, have been identified in self-limited HCV patients[87], vaccines containing these epitope regions are currently being studied (**Section 4**).

The development of therapeutic vaccines against HCV is challenging, not only because such vaccines have to tackle the broad range of HCV types, but also because there is a lack of appropriate animal models and, most importantly, vaccination strategies have to deal with the ineffective host immune response against HCV, as discussed above. The high viral production rate and the presence of multiple HCV quasispecies impede the design of an effective universal therapeutic vaccine. The lack of immunocompetent small-animal models for HCV infection stands in the way of evaluation of the efficacy of vaccine candidates. Yet, recently an immunocompetent transgenic mouse has been generated expressing human CD81 and occludin thereby facilitating HCV cell entry[88]. This transgenic mouse could represent a crucial step in the development of an HCV-susceptible immunocompetent mouse model in the near future. And, as discussed in **Section 3.2**, co-administration of a therapeutic vaccine with immune-modulators and/or blockers of inhibitory molecules may potentially restore ineffective immune response.

4. SPECIFIC APPROACHES IN THE DEVELOPMENT OF A THERAPEUTIC VACCINE AGAINST HCV INFECTION

4.1. Peptide- or protein-based vaccines

Vaccines composed of viral peptides or recombinant viral proteins can be generated

relatively easily and are being developed for infectious diseases and cancer. As peptide/protein vaccines in general are not very immunogenic, these vaccines are often combined with adjuvants. And to avoid the risk of immune escape, these vaccines often contain multiple epitopes for the induction of broad CTL and Th responses.

4.1.1. Preclinical studies

To induce an HCV-specific cellular immune response, already in 1999 Hiranuma *et al*. reported on a study on the vaccination with HCV peptides containing CTL and $CD4^+$ helper T (Th) epitopes[89]. To demonstrate the important role of Th epitopes in activation and maturation of CTL, mice were immunized with either CTL peptides only, a mixture of Th and CTL peptides or conjugated Th-CTL peptides. Th-mediated enhancement of HCV-specific CTL responses were only observed in the last group demonstrating the indispensable role of conjugated Th peptides for priming of a $CD8^+$ T-cell response[89].

Vaccination with recombinant HCV viral proteins has been reported to induce a Th2-biased response, which favors the production of IL-4 and antibodies rather than a Th1-biased response which favors the production of IFN-γ and activation of CTLs[90]. To induce a balanced immune response, Th1 adjuvants and/or immune modulators have been included in experimental vaccine formulations. Adjuvants such as Montanide ISA 720 or TLR agonists (TLR3, TLR9 or TLR4) shift the HCV-specific immune response in vaccinated mice from a Th2 to a Th1 phenotype[90-92]. Currently, studies on the design of vaccines based on proteins, peptides, DNA and dendritic cells (DC) strongly focus on the selection and/or development of proper adjuvants and/or immune modulators.

4.1.2. Clinical studies

Peptide-based vaccination in clinical trials may either be predesigned[93,94] or personalized[95,96]. Predesigned peptides, which contain immunodominant regions of HCV in specific HLA genotypes, stimulate naïve and resting T cells in healthy individuals and HCV-infected patients. Personalized peptides are tailor-made and stimulate memory T cells and activated T cells that are already present in the patient. To identify effective peptides, peripheral blood mononuclear cells (PBMC) are isolated from the patient and cultured in the presence of different predicted HLA-restricted immunodominant peptides. Only peptides that can activate CTLs are administrated to the patients.

The efficacy of a personalized peptide vaccine derived from the HCV E1, E2, NS3 and NS5A regions has been evaluated in a phase I clinical study[95]. $HLA\text{-}A24^+$ patients with a chronic HCV infection, who did not respond to the standard-of-care therapy, were treated with 14 subcutaneous (s.c.) injections biweekly of a personalized

peptide vaccine in Montanide ISA 51 VG. The treatments were well-tolerated and there was no severe toxicity. These personalized vaccines stimulated peptide-specific IFN-γ production by CTLs in 50% of the vaccinees. However, a decrease of HCV RNA in serum was observed in only 3 out of 12 patients.

In a recent phase I clinical study with personalized peptides, HCV-infected patients were vaccinated with a peptide derived from the HCV core protein supplemented with Montanide ISA 51 VG[96]. This peptide was tested in patients with different HLA genotypes as it contains a CTL epitope[79,80] which is conserved in various HCV genotypes and has binding activity to several HLA class I-A molecules (HLA-A2, HLA-A24, HLA-A26, HLA-A31 and HLA-A33). Peptide-specific CTL responses were enhanced after six vaccinations in 15 out of 25 patients and there was a > 1 log decline of HCV serum RNA level in two patients. The amount of peptide-specific IgG was significantly increased in more than 50% of the vaccinees in both studies using personalized peptides. This augmentation has been described to positively correlate with increased survival of advanced cancer patients[97-99]. Of note, vaccination with personalized peptides activates responses against specific epitopes but this strategy is relatively time-consuming and not applicable for all patients.

Klade and colleagues[93] reported on a phase I clinical trial with a pre-designed peptide vaccine, IC41, containing five synthetic peptides derived from core, NS3 and NS4 of HCV with poly-$_L$-arginine as an adjuvant. The peptides encompassed four HLA-A*0201-restricted CTL epitopes and three CD4 Th epitopes[82]. The study indicates that both healthy volunteers and patients with a chronic HCV infection respond to IC41 vaccination. However, patients with a chronic HCV infection showed a lower induction of epitope-specific CTL activity, proliferation of $CD4^+$ Th cells and production of IFN-γ by epitope-specific $CD4^+$ and $CD8^+$ T cells compared to the healthy volunteers[93]. Because of this suboptimal immune response, which is most likely the result of the low immunogenicity of synthetic peptides, the authors suggest to optimize T-cell responses by including more antigenic peptides or by combining IC41 treatment with the standard-of-care in the future.

An effective therapeutic vaccine also aims at maturation of memory $CD8^+$ T cells which are classified as either effector memory T cells (T_{EM}) or central memory T cells (T_{CM}) based on their location, functions and different expression of surface molecules[100]. T_{EM} cells have been described to stably produce perforin and granzyme B, molecules required to lyse virus-infected cells[101]. Of note, HCV-specific T_{EM} cells dominate in patients with an acute HCV infection, while T_{CM} cells dominate in patients with a chronic HCV infection. Further analysis of the phenotypes of HCV epitope-specific $CD8^+$ T cells in vaccinated patients showed a shift from T_{CM} cells to T_{EM} cells after IC41 vaccination[94]. However, the T-cell responses were too weak to induce reduction of HCV RNA in the serum of most of the patients, which may be due to the low immunogenicity of the peptides[93]. Recently, to optimize

immunogenicity, different vaccination routes and dosages of IC41 have been investigated in healthy subjects. Intradermal (i.d.) administration of IC41 induced more robust peptide-specific immune responses than s.c. administration. Although intradermal vaccination with IC41 proved to be safe, efficacy needs to be enhanced in order to obtain sufficient protective cellular immune responses in patients with chronic HCV infection[102].

Vaccinations with peptides together with adjuvant in general are well-tolerated, even after repeated injections. And although most of the HCV peptide/protein vaccines clinically evaluated so far do induce cellular immune responses, these responses do not seem strong enough or do not encompass the appropriate phenotype of cells that would be required to induce viral clearance in patients with a chronic HCV infection.

4.2. DNA vaccines

In the early 1990s, it was shown that plasmid DNA can directly transfect mouse muscle cells *in vivo*[103]. Upon intramuscular (i.m.) administration to mice, plasmid DNA expressing the influenza A virus nucleoprotein (NP) elicited both antigen-specific antibodies and CTLs[104]. These studies initiated the development of DNA vaccines against infectious diseases and tumors[105].

4.2.1. Preclinical studies

Plasmid DNA encoding antigenic HCV protein(s) or peptide epitope(s) of varying sizes can induce both humoral and cellular immune responses *in vivo*. Antigen-specific memory responses have also been described in animal studies[106,107]. Yet, plasmid DNA injected i.m. or s.c. is poorly immunogenic and several studies have demonstrated that immune induction is lower in non-human primates than in mice[108-110]. In order to stimulate the *in vivo* transfection rate, different immunization approaches such as the usage of a gene gun[111], micro-needles[112], gene-electrotransfer[113] and *in vivo* local electroporation[114,115] have been developed and tested in experimental animal models. All these approaches enhance both humoral and cellular immune responses against expressed HCV viral proteins when compared with conventional i.m. or s.c. injection.

Host immune responses against HCV viral proteins in experimental mice models can also be skewed and enhanced by co-administration of adjuvants such as CpG[116,117], QuilA[117] and/or immune modulators such as Flt3-L and GM-CSF[118], IL-2 (ref. 107) or IFN-α[119]. Mice vaccinated with these adjuvants or immune modulators have increased HCV-specific humoral and cellular immunity. Interestingly, inclusion of sequences of the core protein of Hepatitis B virus, thus creating a priming environment by recruiting "healthy" heterologous T cells and by activating innate signaling, resulted in a restoration of HCV-specific responses in mice tolerant for HCV NS3/4A[120]. Furthermore, co-administration of IFN-α, IL-2 and combinations

of Flt-3L and GM-CSF with HCV DNA vaccines have been shown to protect mice from development of HCV-antigen-expressing tumors. Long-term memory responses are induced in mice vaccinated with HCV DNA vaccine together with a plasmid expressing IL-2 (ref. 107). Some HCV viral proteins are known to directly suppress the host immune system[121]. Modification of these proteins without removal of the antigenic epitopes could also enhance the immune responses against these antigens. Modification of antigens expressed by DNA vaccines, such as truncated core proteins[115], truncated or secreted forms of the E2 protein[122], and codon-optimized NS3/4A proteins[111] has been shown to improve the immunogenicity of these proteins when compared with that of native viral proteins. The main advantage of the use of DNA vaccines is the high flexibility of the approach thereby allowing combinations of strategies as mentioned above, to further improve the overall efficacy of DNA vaccines.

4.2.2. Clinical studies

The first therapeutic HCV vaccine based on plasmid DNA evaluated in a phase I clinical study was CIGB-230. This vaccine is composed of a combination of recombinant HCV core protein and a plasmid DNA expressing HCV core, E1 and E2. The trial was performed in patients with a chronic HCV infection who did not respond to standard-of-care therapy[123,124]. Participants were immunized six times with CIGB-230 by i.m. injections with 4-week intervals. Moderate adverse effects such as headache were observed and no autoimmune responses, which could be possibly induced by DNA immunization[125-127], such as anti-mitochondrial or anti-nuclear antibodies were generated. Almost half of the tested subjects had increased proliferation of $CD4^+$ and $CD8^+$ T cells upon *in vitro* antigen stimulation of PBMCs and increased titers of antibodies against E1 and E2 proteins in the serum. Although only 1 out of 15 vaccinated patients had a reduced level of serum HCV RNA, more than 40% of them had improved liver histology. The phase I trial showed CIGB-230 to be safe for humans. However further studies with optimized vaccination dose schedules and methods of vaccine delivery are required.

ChronVac-C® (ChronTech Pharma AB), which contains a plasmid expressing the NS3/4A proteins of HCV genotype 1 has passed a phase I clinical trial and is now under investigation in a phase II clinical trial. In the phase I clinical study, patients with a chronic HCV infection without previous treatment were immunized i.m. twice with a 4-week interval with ChronVac-C® in combination with *in vivo* electroporation using Inovio's Medpulser® DNA Delivery System. Standard-of-care therapy was given to all participants after the second administration of ChronVac-C®. Results showed that 83% of the participants (5 of 6 patients) achieved a sustained virological response. Standard-of-care normally results in a sustained virological response of 40-50% in this patient group. Yet, as the number of patients included

in this ChronVac-C® trial was limited to six, no firm conclusions can be drawn so far. A phase II clinical trial with an increased number of patients is now underway to evaluate the safety, tolerability and efficacy of ChronVac-C® (ClinicalTrials.gov Identifier: NCT01335711).

4.3. Viral vector vaccines

Similar to DNA vaccines, viral vector vaccines encode target proteins or peptides. The major advantage being that in general viral vector vaccines are more immunogenic than DNA-based vaccines. The major disadvantage is that as these vaccines are derived from viruses, they require extensive evaluation by regulatory authorities, which may delay or even obstruct the progress of clinical application. The mostly tested viral vector vaccines against HCV are based on replication-defective adenovirus and modified non-replicative vaccinia virus Ankara (MVA).

4.3.1. Adenovirus vectors

Among the serotypes of Adenovirus (Ad) vectors investigated, Ad5 induces the strongest and longest lasting humoral and cellular immune response in mice[128]. However, a significant proportion of the world population has pre-existing humoral and cellular immunity against Ad5, with up to 50% in the United States and 88% in South Africa[129-131]. To overcome pre-existing immunity, viral vectors based on low prevalence Ad serotypes such as Ad6 (ref. 132), Ad24 or Ad35 (ref. 133) or on adenovirus strains from chimpanzees (ChAd)[132] with a prevalence of 12%[134] are being developed. Ad6 induces strong humoral responses in Rhesus monkeys, although the effects on cellular immune responses are unknown[135].

Apart from pre-existing Ad immunity, the efficacy of Ad-based vaccines and MVA-based vaccines, which will be discussed in the next subsection, is also diminished in homologous prime-boost immunization protocols due to the induction of neutralizing antibodies against the viral vector initiated by the prime immunization. Heterologous prime-boost immunizations may solve this problem as has been shown by combinations of different serotypes of Ad vectors[133], plasmid DNA and Ad vector[136-138], peptides and Ad vector[139], liposomes and Ad vector[140] or other viral vectors including Semliki Forest virus (SFV)[138], ovine atadenovirus[141], MVA[142] and Ad vector.

Although an adjuvant is normally not required in viral vector-based vaccines, the polarity and breadth of the immune response can be further improved by giving different immune modulators before, during and/or after the immunization. Studies combining an Ad vector expressing HCV proteins and MHC class II chaperone protein invariant chain[143], IL-12 (ref. 136, 140), anti-CD137 antibodies[144] or P60 (inhibitor of Foxp3)[145] have shown dramatic increases in both humoral and cellular responses in mice. The safety and efficacy of these immune modulators in humans

is not yet known.

Recently, Barnes *et al*. developed an HCV vaccine in which Ad6 and ChAd3 vectors were being used to induce a broad HCV-specific T-cells response[132]. A phase I study was performed in healthy human volunteers. Robust production of IFN-γ was induced 2 weeks after the prime immunization with an average of > 1000 IFN-γ-producing cells per 1 million PBMCs at the optimal dose of recombinant virus. This vigorous production of IFN-γ by HCV-specific T cells has never been observed in other clinical trials in which on average 100-500 IFN-γ-producing cells per 1 million PBMCs have been observed. This HCV-specific T-cell response is shown to be long lasting (at least 1 year) with wide diversity. Most importantly, the HCV-specific T cells have a mixed effector/central memory phenotype and have strong effector functions such as degranulation and production of IFN-γ, TNF-α. Of note, the vaccinations induced the production of neutralizing antibodies against the Ad vector which might affect the overall response of HCV-specific T cells. Robust HCV-specific immune responses can be induced in healthy volunteers but the efficacy of these vaccines is not known in patients with persistent HCV infection. Further studies on patients with chronic HCV infection are now underway aiming at the restoration of dysfunctional T cells and, at the same time, broadening of the HCV-specific T-cell response[132].

4.3.2. Modified vaccinia virus Ankara vectors

Similar to Ad vectors, vaccines based on modified vaccinia virus Ankara (MVA) are safe in humans. However, pre-existing immunity and neutralizing antibodies against the vector backbone also reduces the efficiency of MVA-based vaccines. The levels of pre-existing immunity against vaccinia virus, as seen in persons previously vaccinated against smallpox virus, seem to be lower than those against Ad5 (ref. 146, 147). And obviously this vaccine-induced pre-existing immunity will disappear as the population that received this vaccine grows older. Several preclinical studies have been performed and showed that MVA-based HCV therapeutic vaccines can induce HCV-specific cellular immune responses and protect mice from for example, NS3-expressing recombinant murine gammaherpesvirus 68 (ref. 142) or recombinant *Listeria monocytogenes* challenge[148].

TG4040 (Transgene) is a viral vector-based therapeutic HCV vaccine that entered phase I and phase II clinical trials. The vaccine is based on MVA expressing NS3, NS4 and NS5B of HCV. Administration of TG4040 to patients with a chronic HCV infection induced no severe adverse effects in a phase I clinical study. Eight out of 15 patients had a reduction in HCV serum RNA and 4 out of the 8 responders had an increased number of HCV-specific IFN-γ-producing cells[149]. A randomized phase II study (HCVac study) involved 153 patients in three treatment groups: (A) standard-of-care alone, (B) combination of the standard-of-care treatment with TG4040 in the same treatment schedule or (C) pre-vaccination with TG4040 before

the standard-of-care. Preliminary data, published on the website of Transgene shown that a reduction of viral load in treatment group C can be detected one week after the start of treatment which is faster than in both other groups (ClinicalTrials.gov Identifier: NCT01055821).

4.3.3. Alphavirus vectors

To overcome problems related to pre-existing immunity against Ad and MVA in humans, other viral vector vaccines such as alphaviruses including SFV, Venezuelan equine encephalitis virus and Sindbis virus, are currently being evaluated for the induction of HCV-specific immunity[139,150-152]. In general, the immune and anti-tumor responses induced by alphavirus-based vaccines seem more robust than those induced by DNA-, peptide-, recombinant protein-, or adenovirus-based vaccines in animal studies[150,153,154]. This can be ascribed to the high-level antigen expression by these vector systems and the low anti-vector responses induced by the viral vectors themselves[155,156] and, possibly most importantly, the activation of multiple innate signaling pathways[157]. An additional advantage of alphavirus-based vaccines relates to the lack of pre-existing immunity in most humans against the native viruses.

4.4. Recombinant yeast-based vaccines

Recombinant *Saccharomyces cerevisiae* is one of the candidates for vaccine development because of its nonpathogenicity in humans and since this yeast can easily be engineered to express multiple proteins of varying size. Furthermore, recombinant yeast cells are able to activate both innate and adaptive immune responses by activating dendritic cells through direct yeast cells-dendritic cells interaction. Interaction of yeast cells with dendritic cells leads to increase antigen presentation and production of pro-inflammatory cytokines such as TNF-α, IFN-γ, GM-CSF, IL-2, IL-6 and IL-12 (ref. 158). Haller and colleagues showed that heat-inactivated *S. cerevisiae* yeast cells expressing HCV NS3 and core protein (GI-5005) induces effective NS3 and core-specific cellular immune responses in both C57BL/6 and BALB/c mice. Activation of cellular immune responses including induction of CTLs and helper T cells protected mice against a challenge with an HCV-expressing tumor[159].

GI-5005 (GlobeImmune) entered a phase I clinical trial in 2007 and results showed that it is well-tolerated and is able to induce significant HCV-specific immune response in patients with a chronic HCV infection (ClinicalTrials.gov Identifier: NCT00124215). The induction of HCV-specific cellular immune responses positively correlated to the reduction of serum viral RNA in vaccinated patients. A phase II trial aiming to investigate the treatment effect of combining GI-5005 and standard-of-care treatment is ongoing. Although not statistically significant, the preliminary results showed that 63% of the patients with combined treatment achieved sustained virological response as opposed to the 45% in patients receiving standard-of-care

treatment alone. Additional patients were recruited in this trial in order to further evaluate the efficacy of GI-5005 in combination with standard-of-care (ClinicalTrials.gov Identifier: NCT00606086).

4.5. Vaccines based on dendritic cells

Vaccines composed of isolated DC, which are modified *ex vivo* to express foreign proteins, are considered very promising[160,161]. As natural antigen-presenting cells, DCs take up antigens and present these to both T cells and B cells. The phenotypes and immunological functions of antigen-loaded DCs can be extensively characterized before administration of the vaccine to the patient. However, tailor-made treatment is expensive and time-consuming. In addition, the availability of functional DCs from patients depends on the severity of the disease, treatment history of the patients and/or other unknown factors[162-164].

4.5.1. Preclinical studies

Bone marrow-derived dendritic cells (BM-DC) from mice have been loaded with HCV antigen by transfection with either plasmid DNA or mRNA encoding HCV nsPs[165,166], infection with Ad vector expressing HCV nsPs[167-171], uptake of either HCV peptides[168], HCV lipopeptides[172] or HCV nsPs-coated magnetic microbeads[173,174]. Transfection of NS5A mRNA by electroporation into BM-DC resulted in the strongest immune response when compared with BM-DC loaded with plasmid DNA encoding NS5A or recombinant NS5A protein[165]. A comparative study between BM-DC loaded with Ad vector encoding HCV NS3 DNA (AdNS3), NS3 peptides or recombinant NS3 protein showed that infection with AdNS3 resulted in the highest level of antigen loading and expression[165,168,170]. The infection rate of Ad vector could be further enhanced by co-administration of AdNS3 and an adaptor molecule CFm40L containing the Coxsackie adenovirus receptor and CD40L, the ligand for CD40 on the surface of DC, thus targeting AdNS3 directly to BM-DC *in vitro*[167].

To stimulate maturation and cytokine production of DC, BM-DC have been treated *in vitro* with immune modulators such as TLR2 antagonist, Pam_2Cys[172] or IL-10-inhibiting peptides resulting in an increased production of IL-12 (ref. 168). Enhancement of immune responses could also be achieved by repeated injection of antigen-loaded and unloaded BM-DC. Alternatively, splenocytes loaded with antigen-coated magnetic microbeads selected by magnetic field were used for *in vitro* immunization[174].

The ratio of subpopulations of DC is one of the main concerns in the development of cell immunotherapy. $CD8\alpha^+$ DC have the highest antigen-presenting ability[175]. However, the expression of CD8α could not be increased by the *ex vivo* cultivation of BM-DC with GM-CSF, IL-4 and/or IL-10, which do increase the expression of CD11c[175]. In order to obtain balanced subpopulations of DC for *in vitro*

loading of antigen, naïve mice were injected with plasmid DNA encoding Flt3-L to expand the populations of DC *in vivo*[176]. DCs pretreated with Flt3-L have a low maturation level and retain their phagocytic properties that enable the *in vitro* uptake of antigen. These antigen-loaded DCs were able to induce robust $CD8^+$ and $CD4^+$ T cells and prevented tumor formation in mice[173,174,177].

4.5.2. Clinical studies

The application of DC therapy for patients with a chronic HCV infection progresses slowly due to contradictory results on the functionality of monocyte-derived dendritic cells (MoDC) from patients with a chronic HCV infection. Results from earlier studies have shown that MoDC from patients have an immature phenotype and deviating cytokine profile compared with those from healthy donors[162]. However, two studies have shown that the maturation status and cytokine-secreting ability of MoDC from patients with a chronic HCV infection are similar to those of MoDC from healthy donors[163,164]. These contradictory results could be explained by differences in the stage of disease and the pretreatment that the patients experienced. For example, ribavirin can suppress the functions of DC *in vivo*[178]. MoDC obtained from patients who did not respond to the standard-of-care therapy showed reduced maturation markers and cytokine production[162], while cells from patients with a chronic HCV infection with mild disease without the standard-of-care therapy have similar phenotypes and activity as those from healthy donors. Thus, treatment with DC can, at present, not be applied in all patients with a chronic HCV infection, since the disease stage and prior treatment affect DC function. Nonetheless, recently, Jirmo and colleagues showed that human monocytes, of which the functions are not affected by HCV infection, can be infected with a lentivirus vector resulting in the activation of both human $CD4^+$ and $CD8^+$ T cells *in vitro*[179]. This study suggests that the use of monocytes as cell therapy warrants further investigation.

Recently, results of a clinical trial with DC treatment among patients with a chronic HCV infection were reported[180]. MoDC from patients were loaded *ex vivo* with six lipopeptides comprising HLA-A2.1 CTL epitopes (3 core, 1 NS3 and 2 NS4B) individually linked to one conventional Th epitope and a lipid moiety, Pam_2Cys. These antigen-loaded MoDCs were injected once i.d. and sequentially intravenously into patients. Patients had no severe adverse effects throughout the study. Furthermore, patients developed IFN-γ responses against the loaded epitopes but also against novel epitopes which were not present in the lipopeptides suggesting that killing of infected hepatocytes and cross presentation of HCV antigen occurred after vaccination. However, no significant changes in serum viral load, anti-core antibodies, level of cytokines and alanine transaminase were observed. Although the magnitude of the immune response was not robust, it can possibly be improved by increasing the dosage of antigen-loaded MoDCs.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

Despite all efforts to develop a therapeutic vaccine against HCV over the last decades, no therapeutic vaccine has yet reached a phase III clinical trial. Most clinical trials so far demonstrate some decline of HCV serum RNA level and antigen-specific immune responses, yet major therapeutic responses have not been observed. Peptide/protein-based vaccines, which are relatively easy to manufacture, require better adjuvants that enhance appropriate responses and at the same time do not elicit too severe adverse effects. DNA-based vaccines require better delivery methods and viral-vector based vaccines face problems associated with anti-vector responses. Similar problems are observed in the development of therapeutic cancer vaccines. In this research field more and more studies are now being conducted in which cancer vaccines are combined with other therapies. With an increasing number of HCV-specific antiviral drugs under investigation[181], a combination of a therapeutic HCV vaccine and novel antiviral drugs may also become the trend of treatment in the future. Since outcome of any treatment is often affected by both viral and host factors, such as, HCV genotype, presence of quasispecies and HLA type of the patients, it would be worthwhile to study correlations between the effects of any treatment with both viral and host factor which may identify the most optimal therapy for each patient.

To enhance the efficacy of vaccination strategies, vaccines could possibly be combined with methods to direct HCV-specific immune effector cells to the liver and at the same time alleviate the immunosuppressive liver environment. Yet, it will be essential that therapeutic vaccines also restore the exhausted immune function of HCV-specific T cells and broaden their epitope-specific diversity, while, also in a therapeutic setting, vaccines should induce neutralizing antibodies.

From preclinical studies in animal models, it appears that the choice of the antigen is an important factor affecting the quality of therapeutic vaccination against HCV. However, the results from these preclinical studies, performed in HCV non-susceptible animal models, are difficult to extrapolate to humans. Therefore, a prediction of the most effective approach and/or target antigens for humans based on these studies cannot be made. An immunocompetent small animal model, susceptible to HCV infection, would be very instrumental to evaluate therapeutic HCV vaccine candidates before their introduction into clinic. Such models will accelerate the development of therapeutic vaccines for HCV infection.

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3

Alphavirus-based Vaccines Encoding Nonstructural Proteins of Hepatitis C Virus Induce Robust and Protective T-cell Responses

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Alphavirus-based Vaccines Encoding Nonstructural Proteins of Hepatitis C Virus Induce Robust and Protective T-cell Responses

ABSTRACT

An absolute prerequisite for a therapeutic vaccine against hepatitis C virus (HCV) infection is the potency to induce HCV-specific vigorous and broad-spectrum T-cell responses. Here, we generated three HCV vaccines based on a Semliki Forest virus (rSFV) vector expressing all- or a part of the conserved nonstructural proteins (nsPs) of HCV. We demonstrated that an rSFV vector was able to encode a transgene as large as 6.1 kb without affecting its vaccine immunogenicity. Prime-boost immunizations of mice with rSFV expressing all nsPs induced strong and long-lasting NS3-specific $CD8^+$ T-cell responses. The strength and functional heterogeneity of the T-cell response was similar to that induced with rSFV expressing only NS3/4A. Furthermore this leads to a significant growth delay and negative selection of HCV-expressing EL4 tumors in an *in vivo* mouse model. In general, as broad-spectrum T-cell responses are only seen in patients with resolved HCV infection, this rSFV-based vector, which expresses all nsPs, inducing robust T-cell activity has a potential for the treatment of HCV infections.

INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of liver disease and the primary cause for liver transplantation in the western world. The World Health Organization estimated that approximately 150 million people worldwide, i.e., around 2.5% of the world population, are chronically infected with HCV. Sixty to 70% of these patients develop chronic liver disease such as liver fibrosis and cirrhosis and 1-5% of these patients develop liver cancer within 10-40 years after infection[1]. The former standard of care treatment, a combination of antiviral agents, pegylated interferon-α and ribavirin, achieved sustained viral response in less than 50% of the patients with chronic HCV (CHC) infected with HCV genotype 1 (ref. 2). Currently, in some countries, this standard of care treatment is being combined with the novel HCV protease inhibitors boceprevir and telaprevir, resulting in a sustained viral response of ~70% in patients infected with HCV genotype 1 (ref. 3, 4). Yet these drugs are not widely available, the drugs are not always tolerated and not all genotypes respond equally to interferon and the antiviral drugs. In contrast to vaccines against hepatitis A and B virus, there is no prophylactic HCV vaccine available, amongst other reasons due to the high variability of the structural proteins of the virus. Therapeutic vaccines, aimed at inducing T-cell responses against the more conserved proteins of the virus are therefore urgently needed. These vaccines can possibly be used as single treatment modalities or can be combined with standard antiviral treatments.

HCV-specific adaptive cellular immunity plays an essential role to control HCV infection, particularly in the induction of functional HCV-specific T cells[5] as also exemplified by the following observations. The presence of NS3/4A-specific $CD8^+$ T cells is positively correlated with a sustained viral response[6-8]. Patients who develop CHC have a narrow-spectrum and low number of HCV-specific T cells in both circulating blood and liver[5]. A low ratio of the HCV-specific $CD8^+$ T cells to the non-specific $CD8^+$ T cells in infected liver leads to an ineffective clearance of virus and a nonspecific inflammation[9]. Next, dysfunctional HCV-specific T cells with reduced IL-2 secretion and cytotoxic activities are being observed in patients with CHC[10,11], and surprisingly, also in patients who recovered from chronic HCV infection by IFN/ribavirin treatment[12]. Moreover, the longer the duration of the exposure of HCV antigen, the deeper the level of T-cell exhaustion[12]. Thus, immunotherapeutic approaches against HCV, should not only induce *de novo* HCV-specific T-cells production but also restore T-cell function.

Several immunotherapeutic approaches are being developed to induce HCV-specific immune responses[13]. Among those approaches, viral vectors induce the most robust immune response in both preclinical and clinical settings. In this study, recombinant Semliki Forest virus (rSFV) vector, which induces strong and long-lasting antigen-specific response[14], was used to develop a therapeutic vaccine against HCV. The nonstructural proteins (nsPs) of HCV have been identified as promising

vaccine targets due to the fact that they are genetically conserved, essential for viral replication and most importantly, immunogenic. In an effort to improve immune responses against the nsPs of HCV, we generated three rSFV constructs encoding either the entire nsPs of HCV (1. NS2'-5B') or parts of these proteins of HCV (2. NS3/4A and 3. NS5A/B'). The *in vivo* efficacy of these rSFV-based vaccines was determined in naïve and tumor-bearing mice.

RESULTS

Characterization of rSFV encoding the entire or the part of HCV nsPs

Aiming to induce immune responses against the entire or part of the HCV nsPs, three rSFV expressing (i) NS2'-5B', (ii) NS3/4A and (iii) NS5A/B' proteins of HCV, were designed and produced (**Figure 1a**). Production and stability of the HCV nsPs synthesized by rSFV infected BHK-21 cells were determined by ^{35}S-methionine pulse labeling (**Figure 1b**). Incubation with rSFVeNS3/4A induced production of the NS3/4A fusion protein (75.9 kDa) and the NS3 protein (70 kDa) by BHK-21 cells. On the other hand, cells incubated with rSFVeNS2'-5B' synthetized five distinct proteins, corresponding to the NS2/3/4A fusion protein (86.9 kDa), the NS2/3 fusion protein (81 kDa), the NS5B' protein (60.8 kDa), the NS5A protein (49 kDa) and the NS4B protein (28.7 kDa). Cells incubated with rSFVeNS5A/B' produced one NS5A/B' fusion protein (109.8 kDa). Cells incubated with control rSFVe or buffer were negative controls. Proteins expression was also determined by western blotting stained with anti-NS3 and anti-NS5A antibodies (data not shown). The newly constructed rSFVs induced abundant expression of HCV nsPs which were stably expressed till 22 hours after incubation with rSFVs.

Frequencies and phenotypes of NS3-specific T cells induced by rSFV immunizations

Next, to verify the immunogenicity of these newly constructed rSFV vaccines *in vivo*, mice were intramuscularly primed and boosted with 5×10^6 purified rSFV or buffer (phosphate-buffered saline (PBS)) with a 2-week interval and the *in vivo* induction of NS3-specific T-cell response was evaluated. Ten days after the last immunizations, NS3-specific CD8$^+$ cells were identified by GAVQNEVTL-dextramer and the phenotype of these cells was studied. The peptide GAVQNEVTL is derived from HCV NS3 and has been identified as a potent cytotoxic T lymphocyte (CTL) epitope presented by the major histocompatibility complex (MHC) class I molecule H-2D^b of C57BL/6 mice[15]. Both rSFVeNS2'-5B' and rSFVeNS3/4A immunizations induced potent NS3-specific CD8$^+$ T-cell responses, yet mice immunized with rSFV encoding only NS3/4A had higher frequencies than mice immunized with rSFV encoding the entire HCV nsPs (rSFVeNS2'-5B': 2.2% ± 0.4% vs. rSFVeNS3/4A: 5.5% ± 0.4%, $P < 0.05$) (**Figure 2a**). The NS3-specific CD8$^+$ T cells were classified into three

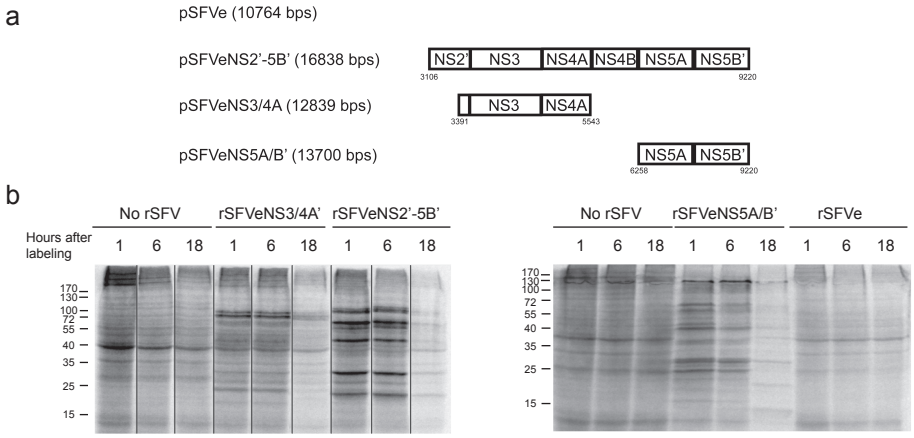

Figure 1. Stable expression of hepatitis C virus (HCV) nsPs *in vitro*. (**a**) Size of plasmid Semliki Forest virus (SFV), number is the nucleotide position in the plasmid DNA containing full genome of HCV 1a (H/FL). (**b**) BHK-21 cells were incubated with rSFVeNS2'-5B', rSFVeNS3/4A, rSFVeNS5A/B' or rSFVe. "No rSFV" was negative control cultured in medium only. Cells were pulsed with ^{35}S-methionine for 1 hour at 6 hours after addition of rSFV and were further cultured for 1, 6 or 18 hours. Radioactively labeled proteins were revealed by autoradiography after 12% SDS-PAGE. The molecular weight of each nonstructural protein is as following: NS2': 11 kDa, NS3: 70 kDa, NS4A: 5.9 kDa, NS4B: 28.7 kDa, NS5A: 49 kDa, NS5B': 60.8 kDa. Vertical lines on the left image show where separate lanes from the same gel were juxtaposed for better comparison with the image on the right. Data represent two independent experiments.

subsets, corresponding (i) central memory T cells (T_{CM}, $CD44^+CD62L^+CD127^+$), (ii) effector memory T cells (T_{EM}, $CD44^+CD62L^-CD127^+$) and (iii) effector T cells (T_{eff}, $CD44^+CD62L^-CD127^-$). The total number of each T-cell subset in the spleen is shown (**Figure 2b**). The increased number of the total NS3-specific $CD8^+$ T cells in mice immunized with rSFVeNS3/4A (**Figure 2a**) was mainly due to the increase in the number of T_{EM} cells and T_{eff} cells ($P < 0.05$). Immunization with rSFV encoding either the entire or the part of HCV nsPs induced NS3-specific responses with similar frequencies of T_{CM} cells.

Effector function of HCV-specific $CD8^+$ cells

In order to investigate the spectrum of the HCV-specific response, spleen cells from rSFV-immunized mice were stimulated with stimulants containing the entire or part of the HCV nsPs and the effector functions of HCV-specific $CD8^+$ T cells were determined. The stimulants were Hepa1-6^V cells, Hepa1-6^V-NS2'-5B' cells, or the HCV synthetic peptides, GAVQNEVTL or ILDSFDPL. Hepa1-6^V-NS2'-5B' cells are murine hepatoma cells expressing HCV NS2'-5B' protein as well as a yellow fluorescence reporter protein, VENUS (abbreviated to ^{V}) and Hepa1-6^V cells are mock transduced cells expressing VENUS protein only (**Supplementary Figure S1**). The peptide ILDSFDPL has been recently identified as a HCV NS5A CTL

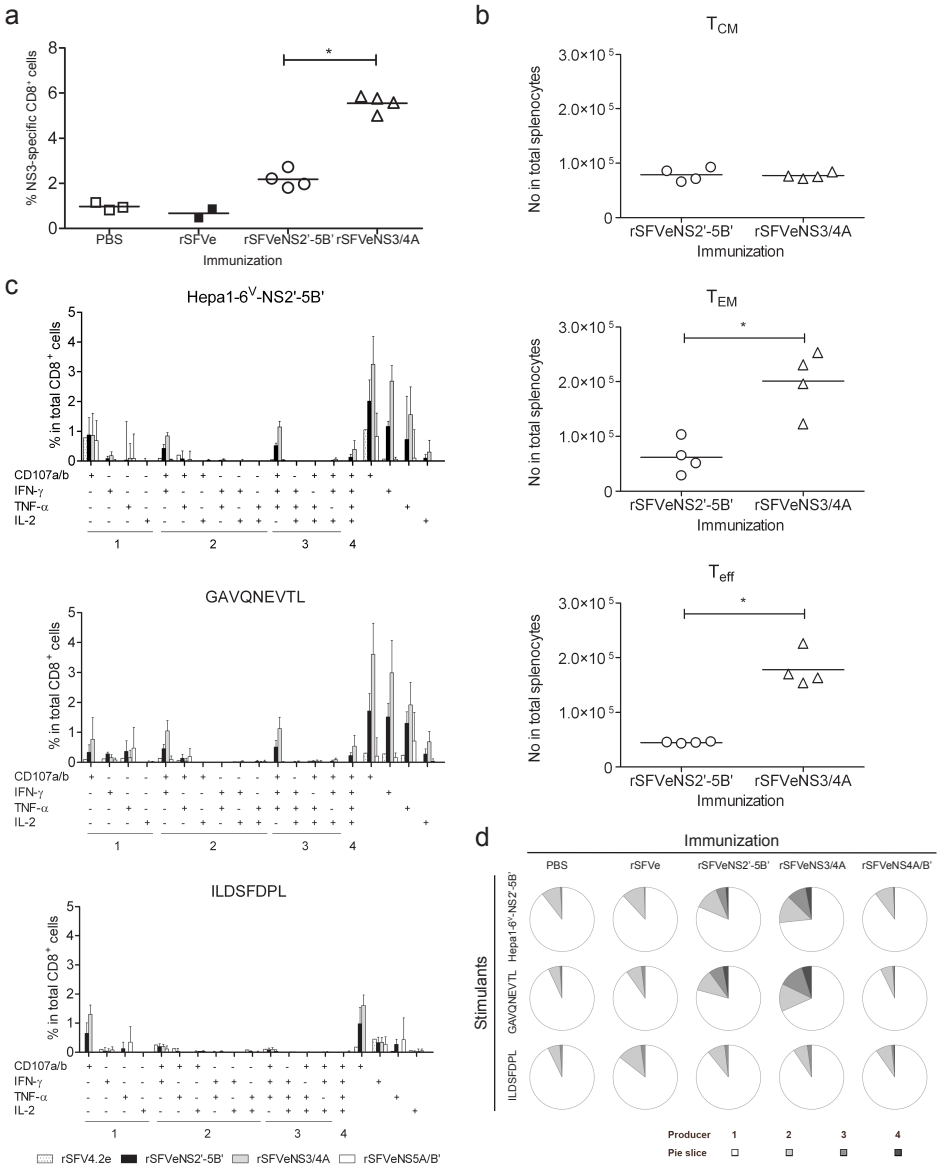

Figure 2. Induction of polyfunctional hepatitis C virus (HCV)-specific CD8⁺ cells by recombinant Semliki Forest virus (rSFV) immunizations. Mice were primed and boosted intramuscularly with 5 × 10^6 rSFV or phosphate-buffered saline (PBS) with a 2-week interval. Ten days after the last immunization, mice were sacrificed and splenocytes were isolated for phenotypic analysis using flow cytometry. (**a**) NS3-specific CD8⁺ cells are shown as a percentage of total CD8⁺ cell population. Data represent two independent experiments showing similar result (n = 2-4). (**b**) Absolute numbers of T_{CM} ($CD44^+CD62L^+CD127^+$), T_{EM} ($CD44^+CD62L^-CD127^+$) and T_{eff} ($CD44^+CD62L^-CD127^-$) cells of the total NS3-specific $CD8^+CD44^+$ cell population in the spleen. (**c**) Splenocytes were stimulated with Hepa1-6^V-NS2'-5B' cells, GAVQNEVTL or ILDSFDPL peptides for 4 hours and subjected to surface and intracellular cytokines staining. The frequencies of each T-cell subset in the CD8⁺ cell population are shown. Background (PBS immunized) subtraction was applied. (**d**) Pie charts present the mean frequencies of T-cell subsets. (**c, d**) 1, single producers; 2, double producers; 3, triple producers; 4, quadruple producers. Data represent the (**a, b, d**) mean and (**c**) mean + SD with n = 2-4. *$P < 0.05$.

epitope presented by the MHC class I molecule, H-2K^b of C57BL/6 mice[16]. The binding affinities of both synthetic peptides were confirmed on RMA-S cells. The GAVQNEVTL peptide bound to H-2D^b at as low as 0.3 μmol/l, while the ILDSFDPL peptide bound to H-2K^b at higher concentration (> 30 μmol/l) (**Supplementary Figure S2**). *In vitro* stimulation with the Hepa1-6^V-NS2'-5B' cells or GAVQNEVTL peptide induced degranulation (CD107a/b$^+$) and secretion of multiple cytokines by CD8$^+$ cells from mice immunized with rSFVeNS2'-5B' and rSFVeNS3/4A (**Figure 2c, 2d**). However, very weak response was detected in mice immunized with rSFVeNS5A/B'. Stimulation with neither Hepa1-6^V cells (**Supplementary Figure S3**) nor ILDSFDPL induced multifunctional CD8$^+$ T cells (**Figure 2c, 2d**). Of note, immunization with rSFVeNS2'-5B' and rSFVeNS3/4A increased the frequencies of polyfunctional CD8$^+$ T cell subsets that produced more than 1 cytokine (*e.g.*, CD107a/b$^+$IFN-γ$^+$TNF-α$^+$ and CD107a/b$^+$IFN-γ$^+$TNF-α$^+$IL-2$^+$ populations) to similar level upon specific stimulations (**Figure 2d**). Lower numbers of NS3-specific CD8$^+$ cells were detected in mice immunized with rSFV encoding the entire HCV nsPs, the effector functions of these HCV-specific CD8$^+$ cells were similar to mice immunized with rSFV expressing NS3/4A only.

Induction of HCV-specific CTL with rSFV immunizations

The most important criterion for functional effector T cells is the ability to lyze their target cells, which, in this study, are the HCV-infected cells. Therefore, the cytolytic activity of HCV-specific CTLs was investigated *in vitro* (**Figure 3b-d**) and *in vivo* (**Figure 3e**). CTL activity was determined 10 days after the last immunization. To detect cytotoxicity *in vitro*, spleen cells isolated from immunized mice were re-stimulated with the Hepa1-6^V-NS2'-5B' cells for 7 days. The number of NS3-specific CD8$^+$ T cells induced after 7-day re-stimulation was higher in mice immunized with rSFVeNS3/4A (58.2% ± 15.8%) than in mice immunized with rSFVeNS2'-5B' (27.6% ± 7.0%) ($P < 0.05$) (**Figure 3a**). This correlated with the higher number of precursor cells in mice immunized with rSFVeNS3/4A (**Figure 2a**). Restimulated CTLs from mice immunized with rSFVeNS2'-5B' or rSFVeNS3/4A lysed Hepa1-6^V-NS2'-5B' cells (**Figure 3b**) and GAVQNEVTL–pulsed EL4 cells (**Figure 3c**) to a similar extent. In general, there was a higher cytotoxic activity against the GAVQNEVTL-pulsed EL4 cells than the Hepa1-6^V-NS2'-5B' cells. The ILDSFDPL-pulsed EL4 cells were not lysed in all groups of mice (**Figure 3d**). Since the cytotoxic effect on Hepa1-6^V-NS2'-5B' cells revealed activity against unidentified HCV nsPs epitopes, this may mimic the real situation during HCV infection when infected liver cells present multi-epitopes. To determine CTL activities *in vivo*, rSFV prime-boost immunized mice were adoptive-transferred with autologous splenocytes pulsed with HCV peptides and irrelevant peptides 10 days after the boost immunizations. The GAVQNEVTL-pulsed autologous splenocytes were lysed in mice immunized with either rSFVeNS2'-

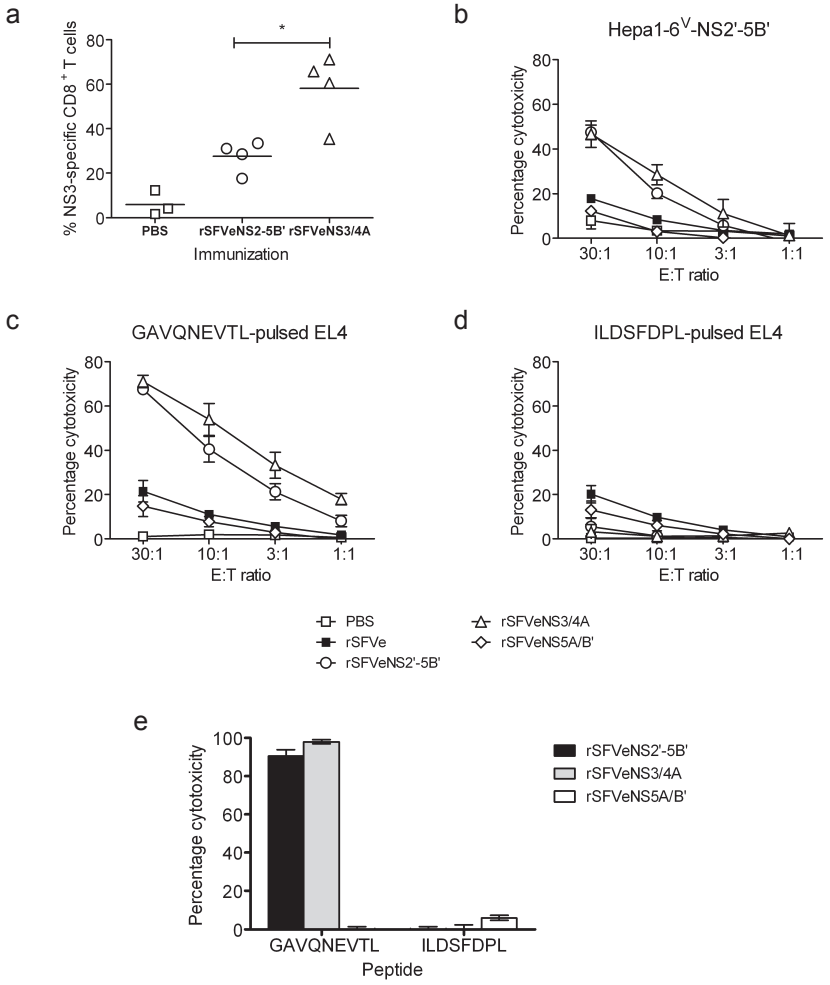

Figure 3. Induction of hepatitis C virus (HCV)-specific cytotoxic T lymphocytes (CTL) with recombinant Semliki Forest virus (rSFV) immunizations. Mice were primed and boosted intramuscularly with 5 × 10^6 rSFV or phosphate-buffered saline (PBS) with a 2-week interval. Ten days after the last immunization, mice were sacrificed and splenocytes were isolated for *in vitro* bulk CTL assay. Splenocytes were cultured with irradiated Hepa1-6^V-NS2'-5B' cells at a ratio of 25:1. After a 7-day culture, splenocytes were subjected to (**a**) NS3-dextramer staining and (**b-d**) bulk CTL assay. Bulk CTL assay was performed by coculturing effector cells, splenocytes, with ^{51}Chromium pulsed target cells at the indicated E:T ratios for 4 hours. Various target cells, (**b**) Hepa1-6^V-NS2'-5B' cells, EL4 cells pulsed with either (**c**) GAVQNEVTL or (**d**) ILDSFDPL peptides, were cocultured with restimulated splenocytes. (**e**) For *in vivo* CTL assay, mice were immunized with the same protocol and were intravenously transferred with peptides-pulsed fluorochromes-labeled autologous splenocytes on day 10 after the last immunization. Each mouse received a mixture of autologous splenocytes containing SIINFEKL-pulsed $CFSE^{lo}$-labeled, GAVQNEVTL-pulsed $CFSE^{hi}$-labeled and ILDSFDPL-pulsed TRITIC-labeled splenocytes at a 1:1:1 ratio. Mice were sacrificed at 14 hours after adaptive transferred. Splenocytes were isolated and the presences of transferred splenocytes were analyzed using flow cytometry. Data represent (**a**) mean and (**b-e**) mean ± SD of two to three independent experiments with n = 2-4. *$P < 0.05$.

5B' (90.5% ± 6.7%) or rSFVeNS3/4 (97.9% ± 2.2%) (**Figure 3e**). No cytotoxic effect on the ILDSFDPL-pulsed autologous splenocytes was detected in all groups of mice. Of note, cytotoxic activity was more potent *in vivo* though no extra re-stimulation of HCV-specific cells was performed. The cytotoxic effector on peptide-pulsed EL4 cells or splenocytes and Hepa1-6 cells expressing all HCV nsPs indicated the potency of these rSFV vaccines inducing functional HCV nsPs-specific CTL *in vivo*.

Therapeutic effect of the rSFV immunizations against EL4 tumor cells

Next, we tested the potencies of these rSFV vaccines to eradicate established HCV nsPs-expressing tumor. Mice subcutaneously inoculated with EL4^{V}-NS3/4A cells, expressing only HCV NS3 and NS4A, were immunized three times with rSFVeNS2'-5B', rSFVeNS3/4A or PBS on days 2, 9 and 16 after tumor inoculation. Mice inoculated with EL4^{V}-NS5A/B' cells, expressing only HCV NS5A and NS5B, were treated with either rSFVeNS2'-5B', rSFVeNS5A/B' or PBS with the same immunization schedule. These EL4 tumor cells express diverse levels of HCV nsPs and VENUS protein mimicking the expression of nsPs by naturally HCV-infected hepatocytes (**Supplementary Figure S4**). With this immunization schedule, the expansion phase of effector T cells after the third immunizations will take place during the exponential growth of tumor cells (days 15-20 after tumor inoculation) as observed in PBS-treated mice. Immunizations with both rSFVeNS2'-5B' and rSFVeNS3/4A induced delay in growth of EL4^{V}-NS3/4A cells ($P < 0.001$) (**Figure 4a**). Of note, immunizations with rSFVeNS3/4A synchronized the rate of tumor growth that was not observed in PBS-treated mice (**Supplementary Figure S5a**). An immunization effect on EL4^{V}-NS5A/B' cells was undetectable (**Figure 4a and Supplementary Figure S5b**).

The effect of immunizations on EL4 tumor cells was further analyzed by analyzing the expression of VENUS protein, being co-expressed with the HCV nsPs. EL4 tumor cells were isolated at different time points when the tumor volume reached ~1 cm^3. VENUS$^+$ cells frequencies were reduced in EL4^{V}-NS3/4A tumor cells in rSFV-immunized mice but not in PBS control ($P < 0.01$) (**Figure 4b**). VENUS expression levels within the VENUS$^+$ cell population (**Supplementary Figure S5c**) and total EL4^{V}-NS3/4A cell population (**Supplementary Figure S5d**) was also reduced. This effect was not time-dependent as expression differences between groups of mice were detected in mice that were sacrificed on the same day. There was no reduction of the number of VENUS$^+$ cells (**Figure 4b**) and the expression level of VENUS in VENUS$^+$ cells (**Supplementary Figure S5c**) and total EL4^{V}-NS5A/B' tumor cells (**Supplementary Figure S5d**) remained the same in all groups of mice. Immunizations with rSFVeNS2'-5B' or rSFVeNS3/4A delayed tumor growth by reducing the HCV NS3/4A expression and the number of VENUS$^+$ cells in the tumor mass.

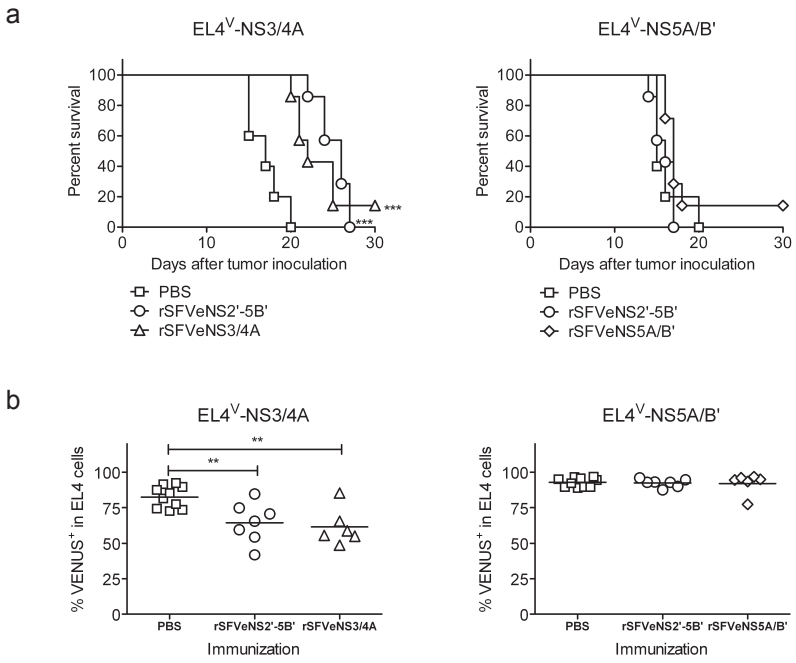

Figure 4. Therapeutic effects of recombinant Semliki Forest virus (rSFV) immunizations against EL4 tumor cells. Naïve mice were subcutaneously inoculated with 5×10^5 of EL4^V-NS3/4A or EL4^V-NS5A/B' cells. Mice were then immunized intramuscularly with 5×10^6 rSFV on days 2, 9 and 16 after tumor inoculation. (**a**) Survival curve is determined when tumor volume equals to 1 cm^3. (**b**) Mice were sacrificed when the volume of tumor exceeded 1 cm^3. Tumor cells were isolated to analyze the frequency of VENUS$^+$ cells in the EL4^V-NS3/4A and EL4^V-NS5A/B' cell population using flow cytometry. Cumulative results of two independent experiments with n = 5-7 are presented. ***$P < 0.001$, **$P < 0.01$.

To investigate the effect of T cells during the early formation of tumor, the first rSFV immunization was performed before tumor inoculation. Mice were immunized with 5×10^6 rSFV particles on day -5, followed with EL4^V-NS3/4A cells inoculation on day 0. Mice were then boosted with rSFV particles twice on day 2 and day 9 after tumor inoculation. The effect on the growth of tumor was mild (**Figure 5a**). Nevertheless, immunizations with rSFVeNS2'-5B' and rSFVeNS3/4A induced reduction on the number of VENUS$^+$ cells (**Figure 5b**) and expression of VENUS in the VENUS$^+$ cell (**Supplementary Figure S6b**) and total EL4^V-NS3/4A cell (**Supplementary Figure S6c**). The immunization effect on EL4^V-NS5A/B' was not determined. In both late and early immunization setups, both rSFVeNS2'-5B' and rSFVeNS3/4A particles reduced the expression of VENUS and the frequencies of VENUS$^+$ EL4 cells demonstrating the activity of HCV-specific CD8$^+$ cells *in vivo*.

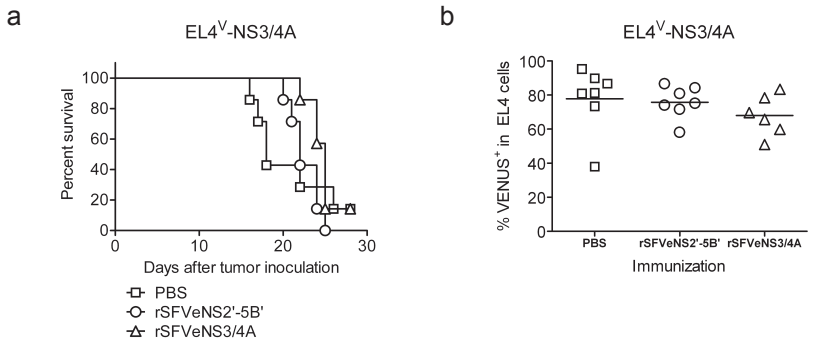

Figure 5. Effect of early immunizations with rSFVeNS2'-5B' and rSFVeNS3/4A against EL4^{V}-NS3/4A tumor cells. Mice were intramuscularly immunized thrice with 5×10^6 recombinant Semliki Forest virus (rSFV) on days -5, 2 and 9. On day 0, immunized mice were subcutaneously inoculated with 5×10^5 of EL4^{V}-NS3/4A cells. (**a**) Survival curve is determined when tumor volume equals 1 cm^3. (**b**) Mice were sacrificed when the volume of tumor exceeded 1 cm^3. Tumor cells were isolated and the frequencies of VENUS$^+$ cells in the EL4^{V}-NS3/4A cell population were analyzed using flow cytometry. Data represent results from one independent experiments with n = 7.

DISCUSSION

Therapeutic vaccination against HCV infection aims to induce robust cellular immunity against conserved HCV proteins leading to eradication of HCV-infected liver cells. Here, we demonstrated that an rSFV-based viral vector vaccine encoding HCV NS3/4A as well as an rSFV vaccine encoding all HCV nsPs induced strong T-cell response against an epitope within the HCV NS3 protein. This NS3-specific T-cell population consisted of both effector and memory cells. Furthermore, these induced NS3-specific T cells were polyfunctional, secreting multiple pro-inflammatory Th1 cytokines and possessed cytolytic activity against HCV nsPs-expressing cells *in vitro* and *in vivo*. Immunizations partially protected mice from HCV NS3/4A-expressing tumor formation. Vaccine-induced immune pressure on the tumors lead to selection of tumor cells, resulting in decreased frequencies of HCV NS3/4A-expressing EL4 cells and reduced expression levels of the transgene (NS3/4A-VENUS).

To our knowledge, this is the first time showing that an rSFV vector retains its immunogenicity when expressing a foreign transgene as large as 6.1 kb. Previously, it has been showed that rSFV expressing a smaller transgene, HCV NS3/4A, induced NS3-specific CTLs[17]. Due to the limitation of the cellular protein synthesis machinery, increasing the size of the transgene will lower the expression of each protein. As a result, vaccine immunogenicity may be reduced or even lost. We demonstrated that an rSFV vector could be packed with all HCV nsPs, expressing all intact HCV nsPs, and induced a HCV NS3-specific response, although this response was lower than the response induced by rSFV expressing HCV NS3/4A only. Thus the immunogenicity of rSFV vector is maintained even when a large transgene is inserted. Increasing the size of foreign transgenes allows broadening of the spectrum of T-cell responses

which is considered essential for the induction of protective HCV immunity[18-20].

T-cell responses against $HCV_{2252-2259}$ ILDSFDPL, an epitope recently identified by Holmstrom, F and colleagues[16], was not detectable in this study. It should however be noted that the ILDSFDPL-specific T-cell responses were identified in mice immunized with DNA plasmid expressing NS5A only. Expressing more than one protein may lead to increase competition between epitopes resulting in reduced responses against subdominant epitopes[21]. Yet to allow a non-HLA restricted application of HCV vaccines expression of as many relevant proteins as possible is desirable. Other T cell epitopes in HCV nsPs were predicted with mathematic algorithms including SYFPEITHI[22], NetMHCpan 2.8 (ref. 23) and Immune Epitope Database and Analysis Resource (IEDB)[24]. The MHC affinity of the identified peptides was determined and the peptides were used to re-stimulate splenocytes from rSFVeNS2'-5B' and rSFVeNS5A/B' immunized mice. Minor T-cell responses against $NS2_{139-147}$, $NS5B_{2-10}$ and $NS5B_{157-165}$ were observed in both groups of mice (manuscript in preparation). Nevertheless, rSFV-based vaccines encoding all possible T-cell epitopes allow induction of a unique spectrum of immune response depending on the patient's HLA alleles.

The magnitude of a T-cell response is positively correlated with the dominance of its cognate epitope[25]. Activation of T cells against a protective but not pathogenic immunodominant epitope results in control of virus-induced diseases[26]. We observed induction of $CD8^+$ T-cell responses against an immunodominant epitope located in the HCV NS3 protein in mice immunized with rSFV expressing either NS3/4A or all HCV nsPs. In the latter group however, lower frequencies were observed. Since the anti-tumor effect of both rSFV vectors was similar, this suggests that T-cell responses against this immunodominant T-cell epitope are protective when a threshold frequency is reached[27].

On the other hand, activation of T cells against one immunodominant epitope facilitates selection of viral escape mutants[28,29]. Furthermore, enhanced responses against immunodominant epitopes will reduce or even abrogate response against subdominant T cell epitopes narrowing the T-cell repertoire[26,30]. Therefore, T-cell responses against subdominant epitopes are desirable[31,32]. Interestingly, we observed a decreased response against the immunodominant epitope ($HCV_{1629-1637}$ GAVQNEVTL) in mice immunized with rSFV expressing all HCV nsPs as lower frequencies of GAVQNEVTL-specific T cells were detected which may indeed allow enhanced responses against other subdominant epitopes[33].

The functionality of *de novo* induced antigen-specific $CD8^+$ cells is also a reliable indicator for effective immunizations[34]. A hallmark of an effective antiviral T cell is the secretion of multiple Th1 cytokines including IFN-γ, TNF-α and IL-2 (ref. 34). IFN-γ and TNF-α mediate control of intracellular infections synergistically[35] while IL-2 strongly enhances the expansion of both $CD4^+$ and $CD8^+$ T cells and

the development of memory cells[36]. Furthermore, it has been shown that IFN-γ reduces the expressing of HCV nsPs *in vitro*[37]. In a recent study, heterologous prime-boost immunizations with a DNA and a MVA vaccine successfully enhanced the number of multifunctional T cells[38]. In our study, with homologous prime-boost rSFV immunizations, function-active HCV-specific T cells secreting IL-2, IFN-γ and TNF-α were induced. In addition, we showed that the HCV-specific T-cell population contained not only effector T cells, but also high numbers of cells with a memory phenotype (both T_{CM} and T_{EM} cells). Central memory T cells have a high proliferation rate and are high producers of IL-2, whereas effector memory cells are potent producers of IFN-γ and cytotoxic granules[39]. Phenotype and cytokine secretion of HCV-specific T cells reflect the potency of rSFV-based vaccines. It would be highly interesting to study the immunogenicity of rSFV vectors in mice or other animal models with exhausted T cells as patients with CHC have dysfunctional T cells[10,11].

Small animal models susceptible for HCV infection are not available yet, and still under development[40]. To investigate the immunogenicity of our rSFV-based HCV vaccines, we therefore produced a polyclonal EL4 tumor cells line by transducing EL4 with HCV nsPs-expressing lentivirus. These tumor cells are heterogeneous, expressing variable levels of HCV nsPs as indicated by the expression level of the cotransduced reporter gene VENUS. Also, in natural HCV infections, this heterogeneity in expression level occurs because of the HCV genetic diversity within a host[41-43]. Eradication of these heterogenic tumors involved negative selection and lysis of the $VENUS^{hi}$ tumor cells by tumor-specific $CD8^+$ T cells. Furthermore, the kinetics of effector $CD8^+$ T-cell responses plays a crucial role on the developing tumor. In this EL4 model, tumor developed exponentially from day 15 to 25 after tumor inoculation. To investigate the therapeutic efficacy of the rSFV-based HCV vaccines, immunizations were scheduled such that the peak of $CD8^+$ T-cell responses overlapped with the exponential growth of the EL4 tumor. Since immunizations were given after tumor inoculation, time for negative selection of $VENUS^{hi}$ EL4 cells was short (approximately from day 6 to day 15). As a result, eradication of tumor cells occurred during the expansion and plateau phases of T-cell response but the reduction of VENUS expression was moderate. In other immunization setups when immunization was given before tumor inoculation, tumor cells were exposed to activated T cells for longer time (from day 0 to day 15) allowing more selection pressure on $VENUS^{hi}$ cells resulting in a significant reduction of the expression of VENUS. However, selection alone was not enough for eradication of this fast-growing tumor as the $VENUS^{lo}$ cells escaped from the immune responses and started to grow exponentially during the contraction phase of T-cell responses. Therefore, rSFV-based HCV immunizations induced immune pressure on HCV nsPs-expressing cells resulting in lower expression of HCV nsPs. Furthermore, the development of the nsPs-expressing EL4 tumor was delayed by the presence of the robust HCV-

specific T-cell responses.

To conclude, rSFV encoding the entire HCV nsPs is a potent vaccine inducing robust and effective HCV-specific immunity. Immune pressure was exerted on HCV nsPs-expressing tumor cells reducing the expression and frequencies of HCV nsPs-expressing tumor cells. The next step will be to study combinations of therapeutic vaccines with other immune interventions and/or direct acting antiviral drugs. For example, PD1/CTLA-4 blockage[44,45] and anti-TIM3 treatment[46] have been shown to restore HCV-specific dysfunctional T cells *in vitro*, while direct acting antiviral drugs aimed to reduce viral load result in reduced production of viral mutants as well as a decreased T-cell exhaustion[47]. Combined treatment of a potent HCV-specific vaccine with immune-interventions and/or direct acting antiviral drugs will hopefully result in *de novo* HCV-specific T cells and restoration of existing dysfunctional T cells resulting in a better prognosis in patients at various stages of HCV infection.

MATERIALS AND METHODS

Cell culture. Baby hamster kidney cells (BHK-21, ATCC #CCL-10) were obtained from the American Type Culture Collection and were maintained in RPMI1640 (Life technologies, Bleiswijk, The Netherlands) supplemented with 10% fetal bovine serum (FBS) (Lonza, Basel, Switzerland). EL4 lymphoma cells and RMA-S cells were kindly provided by Cornelis JM Melief (Leiden University Medical Center, The Netherlands) and were maintained in IMDM (Life technologies) supplemented with 10% FBS. Hepa1-6 cells (provided by Jurgen Seppen, Academic Medical Center, Amsterdam, The Netherlands) and Human embryonic kidney (HEK) 293T cells were maintained in DMEM (Life technologies) supplemented with 10% FBS. All cells were cultured with 100 U/ml penicillin and 100 μg/ml streptomycin (Life technologies) at 37°C with 5% CO_2 otherwise indicated.

Construction of rSFV replicon vectors. pSFV-helper 2 and pSFV4.2 (pSFV), were provided by P Liljestrom (Karolinska Institute, Stockholm, Sweden). The plasmid DNA containing the full-length cDNA of HCV H77 genotype 1a consensus sequence (H/FL) was kindly provided by Charles M Rice via Apath, LLC (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: p90HCVconsensuslongpU)[48]. pUC57-enh which contains the translational enhancer, foot-and-mouth disease virus 2A auto-protease fragment, was synthesized by Eurogentec (Maastricht, the Netherlands). pSFVe (10764 bps) was generated by subcloning the translational enhancer from pUC57-enh into pSFV between the BamHI and BssHII sites. pSFVeNS2'-5B' (16838 bps) was generated by subcloning NS2'-5B' from H/FL into pSFVe between the BssHII and NotI sites. pSFVeNS3/4A (12839 bps) was constructed by cloning the BssHII-NS3/4A-SpeI fragment, which was amplified by PCR using H/FL as a template DNA, into pSFVe between the BssHII and SpeI sites. pSFVeNS5A/B' (13700 bps) was constructed by cloning the BssHII-NS5A/B'-NotI fragment, which was amplified by PCR using H/FL as a template DNA, into pSFVe between the BssHII and NotI sites. All restriction enzymes were purchased from Thermo Fisher scientific (Landsmeer, The Netherlands). DNA sequences were verified by sequence analysis.

Production, purification and titer determination of rSFV. rSFVe, rSFVeNS2'-5B', rSFVeNS3/4A and rSFVeNS5A/B' were produced as previously described[49]. In brief, the

plasmid DNA was *in vitro* transcribed into RNA using SP6 RNA polymerase (GE Healthcare, Diegem, Belgium). RNA of pSFV encoding various parts of HCV nsPs and pSFV-helper2 were co-transfected at a molar ratio of 1:1 into BHK-21 cells. Likely due to the size of rSFVeNS2'-5B', lower production of rSFVeNS2'-5B' was observed. Therefore, transfected BHK-21 cells were cultured at 30°C with 5% CO_2 for 78 hours instead of 37°C for 36 hours as described previously. The supernatant containing the viral particles was collected and tittered with BHK-21 cells. Titer of unpurified rSFVeNS2'-5B' was ~2×10^7 particles/ml and titers of other rSFV particles were ~1×10^8 particles/ml. rSFV particles were further purified on a discontinuous sucrose density gradient and titrated with BHK-21 cells.

Production of HCV nsPs-expressing lentivirus and HCV nsPs-expressing cell lines. Packaging construct (pCMV 8.91), Glycoprotein envelop plasmid (pMD2.G) and lentiviral vector expressing VENUS (442 New pRRL.PPT.SF.IRES-VENUSSnucmer pre or pLenti) were kindly provided by Jan Jacob Schuringa (University of Groningen, The Netherlands). pLenti-NS2'-5B'-VENUS was generated by subcloning the BglII-NS2'-5B'-BglII fragment from pSFVeNS2'-5B' into the BamHI site of pLenti vector. pLenti-NS3/4A-VENUS was generated by removing the AgeI-NS5A/B'-Eco81I fragment from pLenti-NS2'-5B'-VENUS. The sticky ends of AgeI and Eco81I were then filled by Klenow fragment and self-ligated. pLenti-NS5A/B'-VENUS was generated by removing Bst1107I-NS2'-4A-Eco81I from pLenti-NS2'-5B'-VENUS. The sticky ends produced by Eco81I were filled in by Klenow fragment and ligated to the blunt ends produced by Bst1107I. To confirm the expression of HCV nsPs, HEK 293T cells were transfected with each plasmid DNA and the expressed nsPs were determined by western blot analysis (data not shown).

Production of lentivirus and transduction of target cells was performed as previously described for other lentiviruses and cells[50]. In brief, HEK 293T cells were transiently transfected using FUGENE HD (Promega, Leiden, The Netherlands) with pCMV 8.91, pMD2.G and pLenti DNA (pLenti-NS2'-5B'-VENUS, pLenti-NS3/4A-VENUS, pLenti-NS5A/B'-VENUS or pLenti-VENUS) at a molar ratio of 3:0.7:3. HEK 293T culture medium was replaced by complete medium of EL4 cells or Hepa1-6 cells 24 hours after transfection. The supernatant containing lentiviral particles were harvested, passed through a 0.45 μm filter and stored at -80°C.

Hepa1-6^V (^{V} represents VENUS), Hepa1-6^V-NS2'-5B', EL4^V-NS3/4A, EL4^V-NS5A/B' cells were generated by transducing Hepa1-6 or EL4 cells with corresponding lentivirus particles. Two cycles of transduction were performed with a 6-hour interval. Forty-eight hours after the second transduction, cells were harvested and the VENUS$^+$ population was isolated by cell sorting using MoFlo Astrios (Beckman coulter, Woerden, The Netherlands). Cell sorting was performed thrice with a 1-week interval to produce stable cell lines. Expression of VENUS was verified using FACSCalibur cytometer (BD Bioscience, Breda, The Netherlands). Fluorescence activated cell sorting (FACS) data were analyzed using FlowJo analysis software (Tree Star, Ashland, OR) otherwise indicated. More than 95% of the sorted cells had stable expression of VENUS after 1 month of culture (**Supplementary Figure S1 and S4**). VENUS expression of each transduced cells was verified before every experiment.

Protein expression by pulse labeling. BHK-21 cells (5×10^5 cells in a well of a six-well plate) were incubated with 5×10^6 rSFVe, rSFVeNS2'-5B', rSFVeNS3/4A or rSFVeNS5A/B'. After 6 hours, supernatant was removed and the cells were washed with PBS 3 times. The cells were then further cultured in methionine-free DMEM for 30 min followed by labelling with

[^{35}S]-methionine (0.37 Mbq/well) (PerkinElmer, Groningen, The Netherlands) for 1 hour. After 1, 6 or 18 hours of [^{35}S]-methionine labeling, cells were washed with cold PBS and then lysed with TENT lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, and 0.5% Triton-X-100, pH7.5) containing 0.2 mM phenyl-methane-sulphonyl-fluoride. Cell lysate was analyzed by SDS/PAGE and autoradiography.

Synthetic peptides. The synthetic peptides $HCV_{1629-1637}$ GAVQNEVTL (H-2D^b), $HCV_{2252-2259}$ ILDSFDPL (H-2K^b), $OVA_{257-264}$ SIINFEKL (H-2K^b) and human papillomavirus-16-$E7_{49-57}$ RAHYNIVTF (H-2D^b) were manufactured by the department of Immunohematology, Leiden University Medical Center, The Netherlands. The purities of the synthetic peptide were analyzed with HPLC. All synthetic peptides have a purity of > 90%.

Peptides stabilization assay. RMA-S cells were cultured at 26°C with 5% CO_2 for 48 hours to induce expression of MHC class I. Cells were then incubated with various concentrations of synthetic peptides at 26°C for 4 hours, followed by cultured at 37°C for 1 hour. Cells were harvested, washed once with 0.5% bovine serum albumin/PBS and stained with APC-anti-H-2K^b Ab (clone: AF6-88.5.5.3) and FITC-anti-H-2D^b Ab (clone: 28-14-8) (eBioscience, Vienna, Austria) at 4°C for 20 min. The surface expression of MHC class I molecules were analyzed by FACSCalibur cytometer (BD Bioscience).

Mice. Specific pathogen-free female inbred C57BL/6JOlaHsd (H-2^b) mice were obtained from a commercial vendor (Harlan CPB, Zeist, The Netherlands) and were kept under the institute guidelines of the University of Groningen, The Netherlands. All mice were 8 to 10 weeks of age at the start of all experiments. All animal experiments (DEC number: 5946) were approved by the local Animal Experimentation Ethical Committee (the Institutional Animal Care and Use Committee of the University Medical Center of Groningen).

Prime-boost immunizations. Mice were intramuscularly primed and boosted immunized with a 2-week interval with 5×10^6 rSFV (rSFVe, rSFVeNS2'-5B', rSFVeNS3/4A or rSFVeNS5A/B') in 50 µl (25 µl/thigh muscle) under anesthesia (isoflurane/O_2). For negative controls, the same volume of PBS was injected intramuscularly.

Phenotypic analysis of NS3-specific cells. Splenocytes and blood cells were stained with PE-GAVQNEVTL-dextramers (Immudex, Copenhagen, Denmark) in 5% FBS/PBS for 10 min at room temperature. Followed by staining with PE-Cy7-anti-CD8a Ab (clone: 53-6.7), PerCP-Cyanine5.5-anti-CD44 Ab (clone: IM7), APC-anti-CD62L Ab (clone: MEL-14) and eFluor 450-anti-CD127 Ab (clone: A7R34) for 20 min at 4°C. All antibodies were purchased from eBioscience. Death cells were excluded with 4'-6'-diamidino-2-phenylindole staining. FACS analysis was conducted with LSR-II flow cytometer (BD Bioscience).

Identification of multifunctional HCV-specific cells. Splenocytes isolated from immunized mice were cultured with the 100 Gy-irradiated Hepa1-6 cells (Hepa1-6^V-NS2'-5B' cells or Hepa1-6^V cells) at a ratio of 25:1 or with 10 µg/ml of synthetic peptides. The Hepa1-6 cells were cultured in the presence of 50 U/ml of recombinant mouse IFN-γ (Peprotech, London, UK) for 48 hours before coculturing with splenocytes. Splenocytes were cultured in the presence of anti-CD28 Ab (clone: PV-1, Bioceros B.V., Utrecht, The Netherlands), eFluor 660-anti-CD107a Ab (clone: eBio1D4B) and eFluor 660-anti-CD107b Ab (clone: eBioABL-93)

in a 96-well plate at 37°C with 5% CO_2. One hour after culture, brefeldin A (1 mg/ml) was added and the cultures were further incubated for 4 hours. Cells were then harvested, washed and stained with LIVE/DEAD fixable violet dead cell stain kit (Life technologies) according to manufacturer's manuals. Followed by surface staining with PE-Cy7-anti-CD8a Ab at 4°C for 20 min and intracellular staining with PerCP-Cyanine5.5-anti-IFN-γ Ab (clone: XMG1.2), FITC-anti-TNF-α Ab (clone: MP6-XT22) and APC-Cy7-IL-2 Ab (clone: JES6-5H4) at 4°C for 30 min. eFluor 660-anti-CD107a, eFluor 660-anti-CD107b and PerCP-Cyanine5.5-anti-IFN-γ antibodies were purchased from eBioscience; FITC-anti-TNF-α and APC-Cy7-IL-2 antibodies were purchased from BD Biosciences. FACS analysis was conducted with LSR-II flow cytometer, and data were analyzed using FCOM tool of WinList software (Verity Software House, Topsham, ME) and presented as pie charts using SPICE version 5.3 (NIAI freeware).

Bulk CTL assay. The stimulator cells, Hepa1-6^V-NS2'-5B' cells, were cultured in the presence of 50 U/ml recombinant murine IFN-γ for 48 hours. Hepa1-6^V-NS2'-5B' cells were then irradiated (100 Gy) and co-cultured with effector cells, splenocytes, at a ratio of 1:25 in a T25 flask at 37°C with 5% CO_2. Recombinant human IL-2 (5 U/ml) (Peprotech) was added on day 3 and day 5 of the culture. After 7 days culture, splenocytes were harvested and co-cultured with ^{51}Chromium (^{51}Cr)-labeled target cells (Hepa1-6^V-NS2'-5B' cells, EL4 pulsed with GAVQNEVTL or EL4 pulsed with ILDSFDPL). Target cells were cultured in the presence of recombinant murine IFN-γ (50 U/ml) 48 hours before co-culture with effector cells. Target cells were labeled with ^{51}Cr (100 μCi/2 × 10^6 cells) (PerkinElmer) in the presence or absence of synthetic peptides (10 μg/ml) for 1 hour at 37°C. Co-culture of effector cells and target cells were performed in 96 well plate at 4 E:T ratios in triplicates at 37°C with 5% CO_2. After 4 hours of culture, supernatants were harvested and analyzed with a RiaStar manual gamma counter (Packard, Meriden, CT). The percentage of cytotoxicity was calculated according to the formula: % specific release = ((experimental release – spontaneous release) / (maximal release – spontaneous release)) count per minute (c.p.m).

***In vivo* CTL assay.** Splenocytes were isolated from naïve syngeneic mice and were pulsed with synthetic peptides at 10 μg/ml in IMDM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin for 2 hours at 37°C with 5% CO_2. The peptide-pulsed splenocytes were then washed and re-suspended in PBS. GAVQNEVTL-pulsed and SIINFEKL-pulsed splenocytes were labeled with 5 μmol/l of CFSE (Life technologies) and 0.1 μmol/l of CFSE for 20 min at 37°C, respectively. ILDSFDPL-pulsed splenocytes were labeled with 30 μg/ml of tetramethylrhodamine-6-isothiocyanate (TRITC) (Life technologies) for 10 min at 37°C. Differentially labeled cells were washed, re-suspended in PBS and combined at a 1:1:1 ratio for intravenous adoptive transfer. Each mouse received 2 × 10^6 cells of each population. After 14 hours, splenocytes were isolated and analyzed using LSR-II flow cytometer. The percentage of cytotoxicity was calculated with the formula: % cytotoxicity = 1 - (% of relevant peptides-pulsed target (GAVQNEVTL or ILDSFDPL) in rSFV immunized mice/ % of irrelevant peptides-pulsed target (SIINFEKL) in immunized mice)/ (% of relevant peptides-pulsed target in PBS-treated mice/ % of irrelevant peptides-pulsed target in PBS-treated mice).

Tumor inoculation. Mice were inoculated subcutaneously in their right flank with 5 × 10^5 EL4^V-NS3/4A or EL4^V-NS5A/B' cells suspended in 0.2 ml PBS. The volume of tumor was measured by caliper. Cylinder tumor is calculated with the formula 0.7854 × width^2 × length (cm^3). Round tumor is calculated with the formula 0.5236 × diameter^3 (cm^3). Mice were

euthanized when the tumor volume reached 1 cm^3.

Tumor cells isolation. Tumor excised from mice was cut into small pieces and suspended in 5 ml of William's E + Glutamax medium (Life technologies) containing 1 mg/ml of collagenase A (Roche applied science, Almere, The Netherlands). Tumor suspension was transferred into a gentleMACS C-tube and homogenized with the program "m_impTUMOR4" with a gentleMACS™ dissociator (Miltenyi Biotec, Leiden, The Netherlands) followed by 30 min incubation at 37°C. Homogenization and incubation were performed twice. After the second incubation, cell suspension was passed through a 70-µm strainer (BD Bioscience), washed once with 0.5% BSA/PBS and analyzed with FACSCalibur cytometer.

Statistical analysis. Differences between two groups were determined with the Mann-Whitney *U* test. Differences between two survival curves were calculated using the log-rank (Mantel-Cox) test. All data were analyzed with GraphPad Prism software (La Jolla, CA). $P < 0.05$ was considered statistically significant.

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CONFLICT OF INTEREST

Toos Daemen and Hans W. Nijman are cofounders of ViciniVax, a spin-off company from the University Medical Center Groningen developing therapeutic cancer vaccines.The other authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

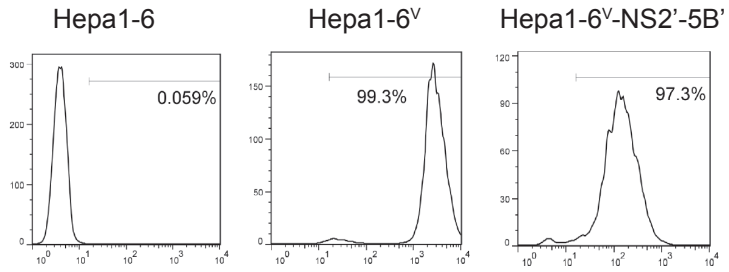

Figure S1. VENUS expression of HCV nsPs-expressing Hepa1-6 cell lines. Hepa1-6 cells were transduced with lentivirus encoding VENUS (represented by ^{V}) or NS2'-5B'-VENUS DNA to produce Hepa1-6^{V} and Hepa1-6^{V}-NS2'-5B' stable cell lines, respectively. VENUS^{+} cell population of each cell line was FACS-sorted thrice resulting stable cell lines containing > 95% of VENUS^{+} cells.

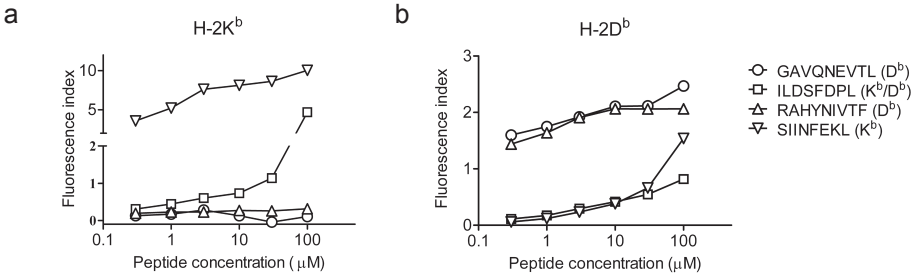

Figure S2. Stabilization of MHC class I molecules on RMA-S cells through binding of HCV peptides. Before incubation with peptides, RMA-S cells were cultured at 26°C with 5% CO_2 for 48 hours to induce MHC class I expression. Cells were then incubated with HCV peptides (GAVQNEVTL, ILDSFDPL) at the indicated concentrations at 26°C for 4 hours, followed by cultured at 37°C for 1 hour. Positive controls, SIINFEKL and RAHYNIVTF are peptides recognizing MHC class I H-2K^{b} and H-2D^{b}, respectively. The expression level of the surface MHC class I molecules, (**a**) H-2K^{b} and (**b**) H-2D^{b}, were analyzed using flow cytometry. Data represent result of three independent experiments.

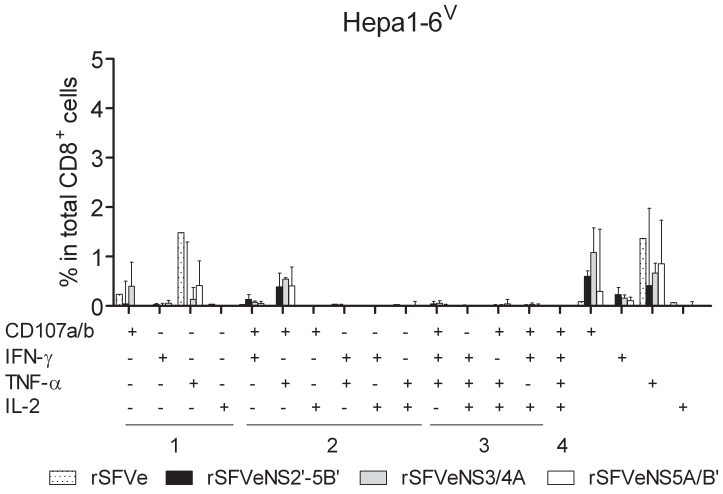

Figure S3. No induction of polyfunctional HCV-specific T cells upon stimulation with Hepa1-6^{V} cells. Mice were primed and boosted intramuscularly with 5 × 10^{6} rSFV or PBS with a 2-weeks interval. Mice were sacrificed on day 10 after the last immunization. Splenocytes were stimulated with the control cells, Hepa1-6^{V} cells, for 4 hours and subjected to surface and intracellular cytokines staining. The frequencies of T-cell subsets in the CD8^{+} cell population are shown. Background (PBS immunized) subtraction was applied. 1, single producers; 2, double producers; 3, triple producers; 4, quadruple producers. Data represent mean + SD with n=2-4.

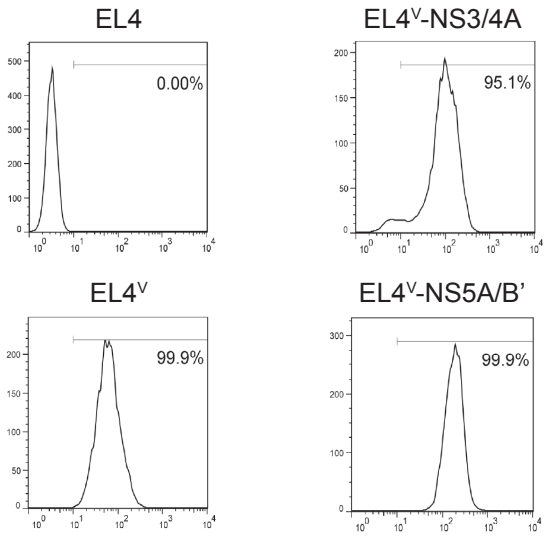

Figure S4. VENUS expression of HCV nsPs-expressing EL4 cell lines. EL4 cells were transduced with lentivirus encoding VENUS, NS3/4A-VENUS or NS5A/B'-VENUS DNA to produce EL4^{V}, EL4^{V}-NS3/4A and EL4^{V}-NS5A/B' cell lines, respectively. VENUS^{+} cell population of each cell line was FACS-sorted thrice resulting stable cell lines containing > 95% of VENUS^{+} cells.

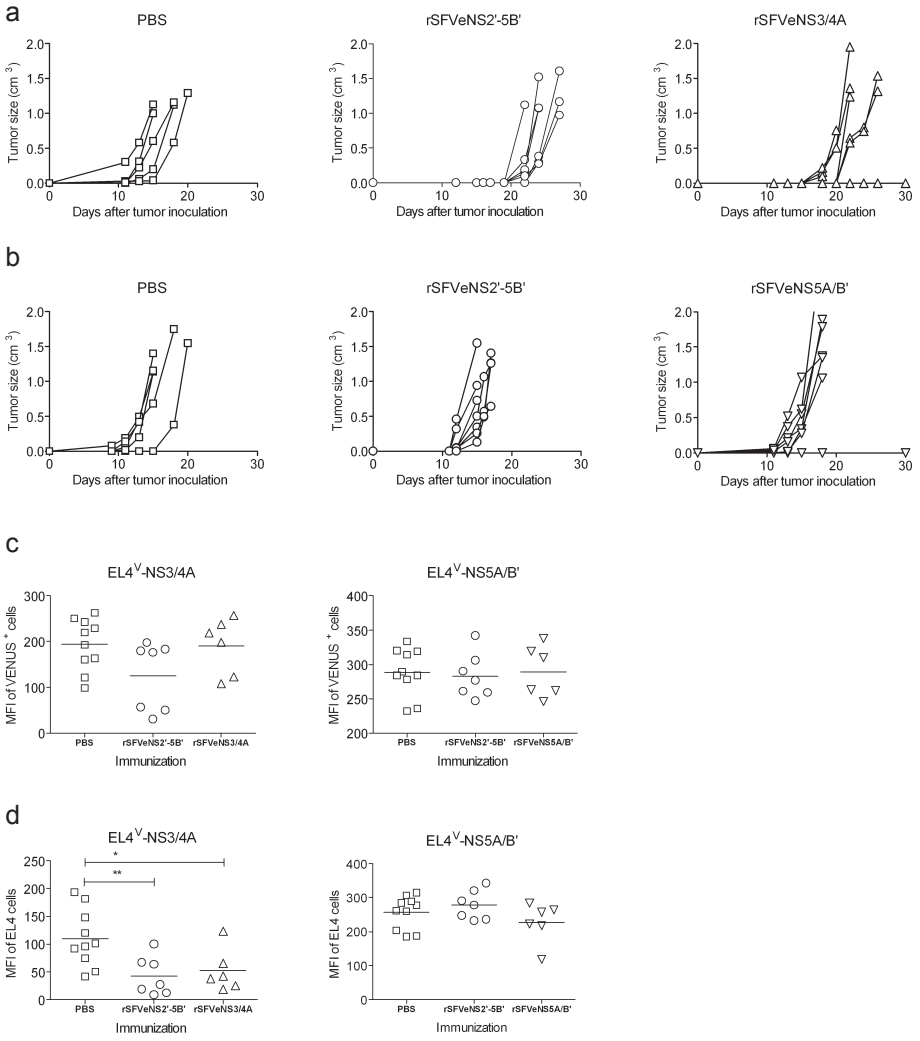

Figure S5. Therapeutic effect of rSFV immunizations against HCV nsPs-expressing EL4 cells. Naïve mice were subcutaneously inoculated with 5×10^5 of EL4^V-NS3/4A or EL4V-NS5A/B' cells. Mice were then immunized intramuscularly with 5×10^6 rSFV on days 2, 9 and 16 after tumor inoculation. Tumor growth curves of each mouse are shown (**a**: EL4^V-NS3/4A cells, **b**: EL4^V-NS5A/B' cells). Mice were sacrificed when the volume of tumor exceeded 1 cm^3. Tumor cells were isolated to analyze the median fluorescence intensity (MFI) of the VENUS$^+$ cells (**c**) and the total EL4^V-NS3/4A and EL4^V-NS5A/B' cell population (**d**) using flow cytometry. Cumulative results of two independent experiments with n = 5-7 are presented. $^{**}P < 0.01$, $^{*}P < 0.05$.

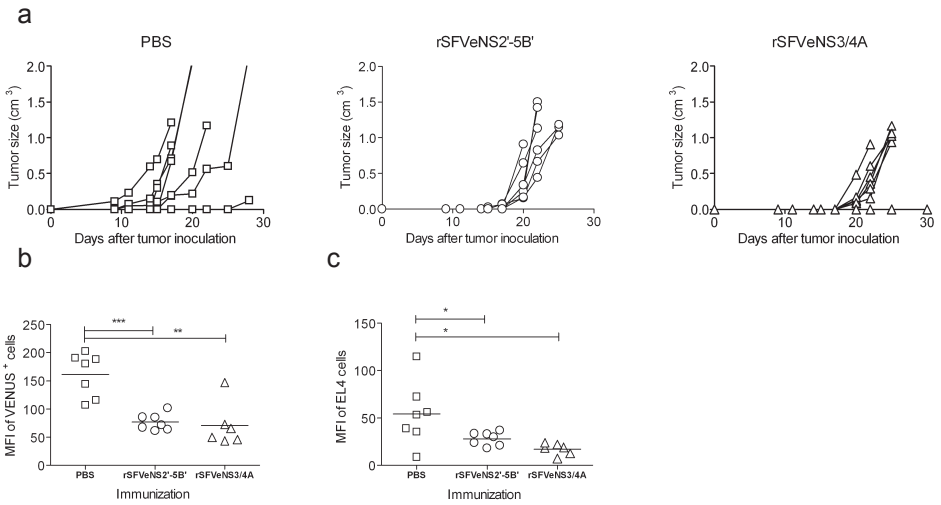

Figure S6. Effect of early immunization with rSFVeNS2'-5B' and rSFVeNS3/4A against EL4^{V}-NS3/4A cells. Mice were intramuscularly immunized thrice with 5 × 10^{6} rSFV on days -5, 2 and 9. On day 0, immunized mice were subcutaneously inoculated with 5 × 10^{5} of EL4^{V}-NS3/4A cells. (**a**) Tumor growth curves of each mouse are shown. Mice were sacrificed when the volume of tumor exceeded 1 cm^{3}. Tumor cells were isolated and the MFI of the VENUS^{+} cells (**b**) and the total EL4^{V}-NS3/4A cell population (**c**) were analyzed using flow cytometry. Data represent results from one independent experiments with n = 7. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

4

Antigen Design Enhances the Immunogenicity of Semliki Forest Virus-based Therapeutic Human Papillomavirus Vaccines

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Antigen Design Enhances the Immunogenicity of Semliki Forest Virus-based Therapeutic Human Papillomavirus Vaccines

ABSTRACT

Cellular immunity against cancer can be achieved with viral vector- and DNA-based immunizations. In preclinical studies cancer vaccines are very potent, but in clinical trials these potencies are not achieved yet. Thus a rational approach to improve cancer vaccines is warranted. We previously demonstrated that the relatively low intrinsic immunogenicity of DNA vaccines could be enhanced by inclusion of endoplasmic reticulum (ER) targeting and universal helper epitopes within the vaccine. We now evaluated whether an optimal antigen format, as defined in DNA vaccines can further enhance the effectiveness of recombinant Semliki Forest virus (rSFV) vaccines. To this purpose, we generated, characterized and evaluated the efficacy of rSFV replicon particles expressing human papillomavirus E6 and/or E7 proteins fused to several helper T cell epitopes and an ER targeting signal. Here we show that inclusion of a helper cassette and an ER targeting signal enhanced protein stability and markedly augmented the frequencies of HPV-specific T cells. Even at an immunization dose of as low as 10^5 replicon particles this novel vaccine achieved tumor regression and protection. Thus, even highly effective viral vector vaccines can benefit from an improved antigen format, based on the inclusion of defined helper epitopes and ER targeting.

INTRODUCTION

Persistent infection with high-risk human papillomavirus (HPV) may result in (pre)malignant cervical intraepithelial lesions that can develop into cervical cancer, the second most common cancer in women worldwide. HPV infection also results in external genital cancers, next to cancer of anus and cancer of mouth and oro-pharynx in both males and females[1]. HPV-induced malignant cells contain the HPV genome in an episomal state and/or a part of the HPV genome integrated into the host chromosome[2-5]. As a result, HPV-transformed malignant cells constitutively express the HPV E6 and E7 oncoproteins, which function cooperatively to facilitate malignant progression[6]. Prophylactic vaccines against high-risk HPV induce protective humoral immunity against the capsid protein L1 (ref. 7, 8). These prophylactic vaccines do not cure patients with persistent HPV infection[9] whereby cellular immunity against E6 and E7 oncoproteins plays a crucial role to eradicate HPV-infected/transformed cells[10-12].

Previously, we demonstrated that a recombinant Semliki Forest virus (rSFV)-based HPV vaccine (rSFVeE6,7) has therapeutic anti-tumor effect in tumor-bearing mice[13,14] and outperformed an adenoviral vaccine encoding HPV antigens[15]. Importantly, in contrast to a protein or DNA-based vaccine, this rSFVeE6,7 vaccine is even immunogenic in HPV16 E6 E7 transgenic mice[16].

Considering the observation that the outcomes of clinical trials with cancer vaccines do not meet the high expectations based on preclinical studies, optimization of vaccine design might further increase robust long-term immune responses, while not increasing or inducing toxicity.

In an effort to improve vaccine potency, carrier proteins have been included in vaccines composed of for example synthetic peptides, recombinant proteins and plasmid DNA[17]. Inclusion of carrier proteins such as FMS-like tyrosine kinase-3 (Flt3) ligands[18], heat shock protein 70 (ref. 19), and tetanus toxin fragment C domain 1 (TTFC)[20] successfully improved the efficacy of HPV DNA vaccines in pre-clinical studies. The mechanism of enhancement has been thought to depend on the biological function of the carrier protein involved[21]. However, in most cases, direct evidence that the biological function of the carrier protein under investigation is responsible for enhanced immunogenicity is limited. In prior work we demonstrated that two vaccine formats containing T-helper (Th) epitopes which can boost the activation of antigen presenting cells (APCs) and thereby enhance $CD8^+$ T-cell responses[22], enhanced E6- or E7-specific T-cell responses in HPV DNA vaccines[20,23]. The two vaccine formats studied included either (i) Tetanus Toxin Fragment C (TTFC), or (ii) the combination of a universal "helper-cassette" and ER targeting signal ($HELP^{ER}$). In the latter vaccine format, a series of Th epitopes, including the TTFC P30 pan DP epitope, PADRE pan DR epitope and HIV NEF pan DQ epitope, is contained[23], and localization and retention of

the antigen within the endoplasmic reticulum (ER) is achieved by inclusion of the human growth hormone signal peptide[24] and a KDEL sequence[25,26]. As compared to vaccination with parental HPV antigen encoding DNA vaccines, immunogenicity of in particular HELP^{ER} HPV DNA vaccines was markedly enhanced[23].

Immunizations with DNA vaccines encoding Th epitopes and ER localization/retention signals induced up to 22% E7-specific T cells within the CD8 population[23]. And remarkably, while rSFVeE6,7 immunizations induced lower percentages of E7-specific T cells (1-4% of CD8$^+$ T cells)[15], the *in vivo* anti-tumor responses were higher (Survival rate at 3 months after tumor inoculation: rSFVeE6,7: 100%; sigHELP-E7SH-KDEL: 50%). Although these *in vivo* results were derived from independent studies in two institutes, this comparison would suggest that the number of E7-specific CD8$^+$ T cells is not the only factor affecting the efficacy of a vaccine[27].

In order to further enhance the potency of rSFV vaccines, we here evaluate the potential added value of the antigen format strategies that proved successful in DNA vaccines. To this end, Th epitopes and ER localization/retention signals-expressing rSFV vaccines were synthesized and characterized, and their ability to induce HPV-specific immune reactivity was investigated in naïve and tumor-bearing mice.

RESULTS

Construction and characterization of rSFV encoding universal T-helper epitopes

To evaluate the effect of external Th epitopes and ER location/retention signals within rSFV, vectors were constructed and characterized *in vitro*. These vectors either expressed an E6,7 fusion protein or a shuffled E7 (E7SH) protein. The HPV E6,7 fusion protein-expressing rSFV (E6,7) induced superior E7-specific response compared to rSFV expressing individual E6 and E7 proteins which could be ascribed to an enhanced stability of the fusion protein[13]. E7SH protein, primarily designed for DNA vaccines, was constructed by removing the retinoblastoma protein (pRb) binding site on E7 by gene shuffling, leading to eradiation of the transforming property of E7 protein[28]. Next to our standard HPV-expressing rSFV construct (1) rSFVeE6,7 (E6,7), the following constructs were generated: (2) rSFVe-E7SH (E7SH), (3) rSFVe-TTFC-E6,7 (TTFC-E6,7), (4) rSFVe-TTFC-E7SH (TTFC-E7SH), (5) rSFVe-sigHELP-E6,7-KDEL (sHELP-E6,7) and (6) rSFVe-sigHELP-E7SH-KDEL (sHELP-E7SH) (**Figure 1a**). The abbreviations in brackets will be used throughout this paper.

Before testing the efficacy of the rSFV replicons *in vivo*, the expression and stability of the transgene-encoded proteins was characterized upon infection of BHK-21 cells. Protein expression was determined in the lysate of BHK-21 cells

24 hours after incubation. All rSFV replicons induced expression of E7 or E7SH protein with the expected molecular weight (**Figure 1b**). As also observed previously, bands of various molecular weights representing high order protein complexes were detected[13]. Inclusion of the helper epitopes, both TTFC and sigHELP-KDEL, enhanced the amount of transgene-encoded product 24 hours after rSFV infection (**Figure 1b**, relative density).

Pulse-chase experiments showed that the E6,7 fusion protein was more stable than the E7SH protein (**Figure 1c**). Furthermore, consistent with the detection of increased amounts of transgene-encoded product, fusion of both TTFC and sigHELP-KDEL to E6,7 or E7SH strongly enhanced the stabilities of the proteins. Even 42 hours after [^{35}S]-methionine labeling, heavily labeled bands were visible (**Figure 1c**).

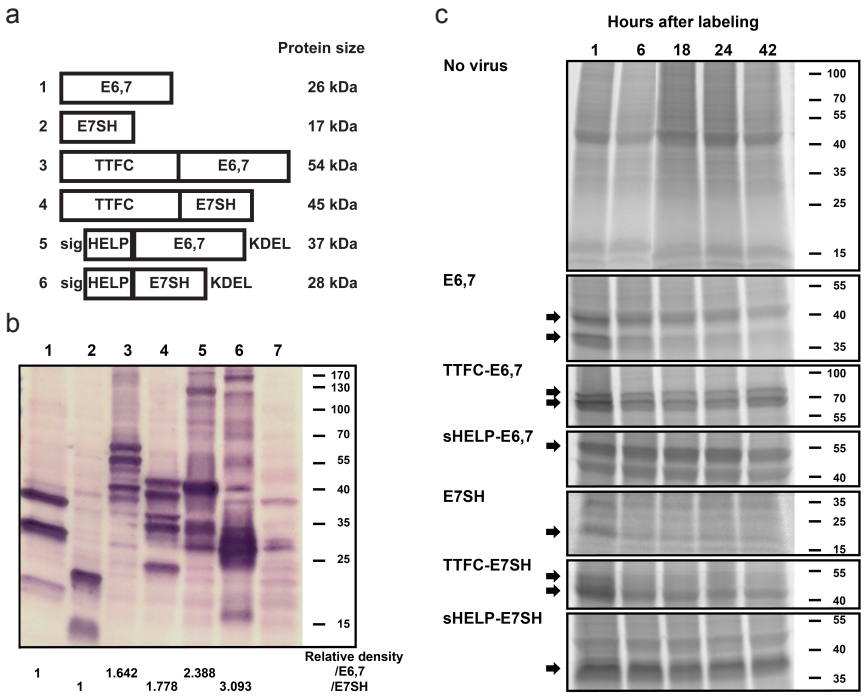

Figure 1. Expression of HPV E7 protein *in vitro*. (**a**) Schematic representation of the rSFV replicons and the size of the fusion proteins. (**b**) BHK-21 cells were incubated with (1) E6,7, (2) E7SH, (3) TTFC-E6,7, (4) TTFC-E7SH, (5) sHELP-E6,7 (6) sHELP-E7SH or (7) without rSFV replicons. After 24 hours incubation, cell lysates were collected and analyzed by SDS-PAGE and immunoblotting. A mouse anti-HPV 16 E7 antibody was used to detect HPV E7 protein. Density of all specific bands were analyzed and the numbers below the western blot indicate the relative density compared to controls (E6,7 or E7SH). (**c**) Proteins were labeled with [^{35}S]-methionine for 1 hour after incubation with rSFV replicons for 6 hours. Cell lysates were collected at 1, 6, 18, 24 and 42 hours post [^{35}S]-methionine labeling for SDS/PAGE and autoradiography. Data represent result from 2 independent experiments.

rSFV immunizations induce E7-specific CD8$^+$ cells

To determine the immunogenicity of the different rSFV replicons, naïve mice were immunized twice with 5 × 10^6 rSFV replicons with a 2-week interval. The frequency of E7-specific CD8$^+$ cells in peripheral blood was measured using $E7_{49-57}$ MHC class I tetramers at different time points. E7-specific CD8$^+$ cells were detected at day 7 after first immunization and boosted by a second injection (**Figure 2**) with a equal induction kinetics in all groups of rSFV-immunized mice. The frequencies of E7-specific cells in total CD8$^+$ cells 7 days after the boost immunization with E6,7 and E7SH were equal. At this time point, genetic fusion of E7SH with TTFC slightly enhanced the frequencies of E7-specific CD8$^+$ cells compared to E7SH alone but no difference was observed in TTFC-E6,7 compared to E6,7 (**Figure 2a**). An increased frequency of E7-specific cells in total CD8$^+$ cells was detected in mice immunized with sHELP-E6,7 compared to E6,7 not only 7 days after the boost immunization (E6,7: 2.8% ± 1.2% versus sHELP-E6,7: 5.6% ± 0.8%, $P = 0.057$) but also at early time points (days 7 , 10, 18 after first immunization, $P < 0.05$). An even stronger effect was observed in mice immunized with sHELP-E7SH compared to E7SH on 7 days after the boost immunization (E7SH: 3.1% ± 0.7% versus sHELP-E7SH: 11.4% ± 5.6%, $P = 0.029$) and also at most of the other time points (days 7, 10 and 25 after first immunization, $P < 0.05$) (**Figure 2b**). Thus while both E6,7 and E7SH induced similar responses, addition of sigHELP and KDEL to E7SH resulted in a stronger augmentation of the E7-specific CD8$^+$ response, i.e., 3.6-fold increase compared to matched control, than when added to E6,7, i.e., 2-fold increase. Nevertheless, even though the magnitude of enhancement differed, inclusion of sigHELP and ER localization/retention signals led to the induction of enhanced CD8$^+$ T-cell responses against both E6,7 and E7SH.

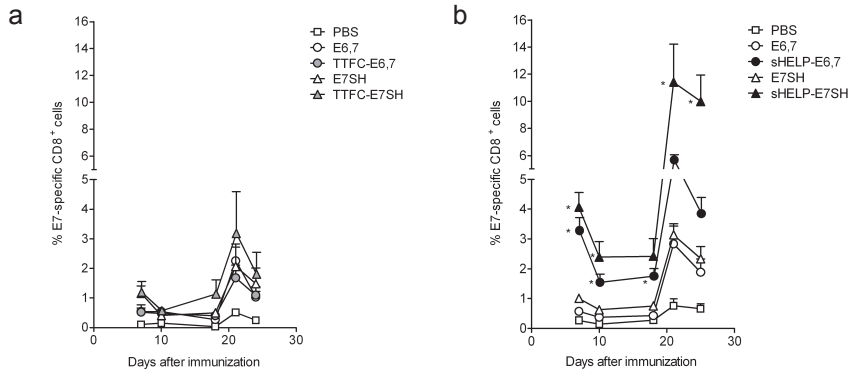

Figure 2. Kinetics of circulating blood E7-specific CD8$^+$ cells upon rSFV immunizations. Mice were primed and boosted intramuscularly (i.m.) with 5 × 10^6 rSFV with a 2-week interval. (**a**) TTFC-expressing rSFV and their matched controls. (**b**) sigHELP-KDEL-expressing rSFV and their matched controls. Blood samples were collected at indicated time points. Percentages of E7-specific cells in CD8$^+$ blood cells are shown. Data represent mean + SEM of each group (n=4) from 2 independent experiments. *$P < 0.05$ (E6,7 versus sHELP-E6,7 or E7SH versus sHELP-E7SH).

Functional capacity of vaccine-induced E7-specific CD8$^+$ cell populations

Since both the quantity and quality of HPV-specific cells are crucial parameters for the effectiveness of an HPV vaccine, we determined phenotype, IFN-γ production capacity and degranulation capacity of vaccine-induced E7-specific CD8$^+$ cells in spleen. Mice were immunized twice with 5×10^6 rSFV with a 2-week interval and were sacrificed 10 days after the last immunization. At this time-point, a trend toward an increased frequency of E7-specific CD8$^+$ spleen cells was observed in mice immunized with sHELP-E7SH to their matched controls (**Supplementary Figure S1a**). While sHELP-E6,7 immunization did not change the central memory T cells (T_{CM}) to effector memory (T_{EM}) ratio as compared to E6,7 immunization, sHELP-E7SH immunization decreased T_{CM}: T_{EM} ratio (**Supplementary Figure S1b**). Yet, also the number of T_{CM} cells did increase upon sHELP-E7SH immunizations relative to E7SH immunizations. The E7-specific CD8$^+$ cells induced with all vaccines, possessed effector cell capacities, as assessed by antigen-induced production of IFN-γ and degranulation (**Supplementary Figure S1c**).

E7-specific CD8$^+$ cells expand and possess cytolytic function after *in vitro* re-stimulation

To further investigate the intrinsic activities of HPV-specific CD8$^+$ cells induced by the different vaccine formats, we re-stimulated splenocytes of vaccinated mice with irradiated TC-1 cells for 7 days and determined the expansion and cytotoxic activity of the resulting HPV-specific cells. In mice immunized with TTFC- or sigHELP-KDEL-expressing rSFVs, there was a trend towards increased E7-specific T cells expansion, as compared to their matched controls (**Supplementary Figure S1d**). The higher cell number may be due to the higher number of precursor E7-specific CD8$^+$ cells in mice immunized with sHELP-E6,7 or sHELP-E7SH before 7-days re-stimulation (**Supplementary Figure S1a**).

Cytotoxic activity of the HPV-specific cells was determined *in vitro* by standard ^{51}Cr release assay using C3 cells expressing the whole genome of HPV 16 as target cells. At higher effector to target (E:T) ratios (30:1 and 10:1), cytolytic activity in all groups of rSFV-immunized mice was similar (**Figure 3**). Only at the lower E:T ratios (3:1 and 1:1), a slightly higher cytolytic activity was detected by splenocytes isolated from sHELP-E7SH-immunized mice compared to their controls (**Figure 3b**). Taken together, these results from both expansion and cytolytic analysis of the E7-specific CD8$^+$ cells suggest that the intrinsic activity of HPV-specific cells induced by all rSFV immunizations remained similar.

Improved therapeutic effect of rSFV vaccines with inclusion of sigHELP-KDEL

Next, we determined the therapeutic effect of rSFV replicon vaccination in mice inoculated with TC-1 tumor cells. Since we did not observe major differences in

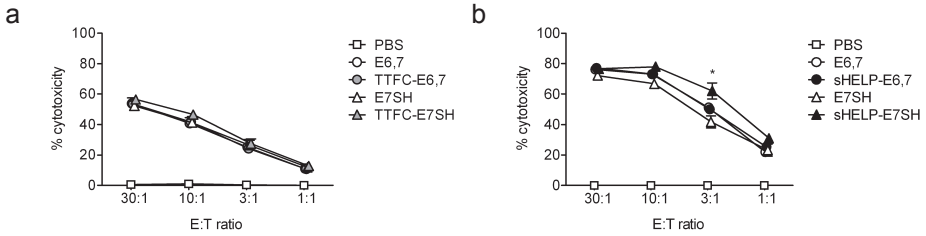

Figure 3. Cytotoxic activity of E7-specific CD8⁺ spleen cells. Mice were primed and boosted i.m. with 5 ×10⁶ recombinant Semliki Forest virus particles with a 2-week interval. Ten days after the last immunization, mice were sacrificed and splenocytes were re-stimulated with irradiated TC-1 cells for 7 days. Bulk CTL assay was performed by co-culturing re-stimulated splenocytes (effector cells, E), with ^{51}-Chromium pulsed C3 cells (target cells, T) at indicated E:T ratios at 37°C for 4 hours. (**a**) TTFC-expressing rSFV and their matched controls. (**b**) sigHELP-KDEL-expressing rSFV and their matched controls. Data represent mean ± SEM of each group (n = 3-4). *$P < 0.05$ (E7SH versus sHELP-E7SH).

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the frequency of E7-specific CD8⁺ cells between rSFVs without carrier proteins and rSFVs expressing TTFC (E6,7 versus TTFC-E6,7 and E7SH versus TTFC-E7SH), the latter vaccines were left out of this analysis. We previously showed that immunizations with 5 × 10⁶ rSFV particles on days 7, 14 and 21 post TC-1 inoculation leads to 100% survival for more than 3 months after tumor inoculation. In order to reveal a possible effect of the carrier proteins, we therefore chose to immunize mice with a suboptimal dose of rSFV (1 × 10⁵ particles) on days 7, 14 and 21 after TC-1 tumor cells inoculation (**Figure 4**). Control mice developed palpable tumors approximately 14 days post tumor inoculation. After that time-point, tumors grew exponentially, reaching a volume of approximately 1 cm³ between day 20 to 25 (**Figure 4a**). Reduced tumor growth rate was observed in mice immunized with a suboptimal dose of all tested rSFV replicons compared to control mice ($P < 0.0001$). Immunization with sHELP-E6,7 not only reduced tumor growth rate but also delayed the formation of tumor (E6,7 versus sHELP-E6,7, $P = 0.0036$). E7SH and sHELP-E7SH immunizations both reduced tumor growth rate and had a similar survival rate. Suboptimal immunizations with sHELP-E6,7, E7SH and sHELP-E7SH shortly after tumor inoculation induced effector T cells and efficiently delayed the growth of tumor to a similar extent (% tumor free mice at 19 weeks post tumor inoculation: E6,7: 0%; sHELP-E6,7: 29%; E7SH: 14%; sHELP-E7SH: 29%) (**Figure 4b**).

rSFV immunizations induce memory response and protect mice from tumor formation

Induction of HPV-specific memory responses is the key to combat HPV re-infection and HPV-associated tumor formation. To investigate the quality of memory T cell responses induced by rSFV immunizations, mice were first immunized twice with a suboptimal dose of rSFV (1 × 10⁵ particles) with a 1-week interval. Next, when the frequency of the E7-specific CD8⁺ cells in blood had declined, i.e., almost 3 weeks after the last immunizations (day 26 after first immunization), tumor cells

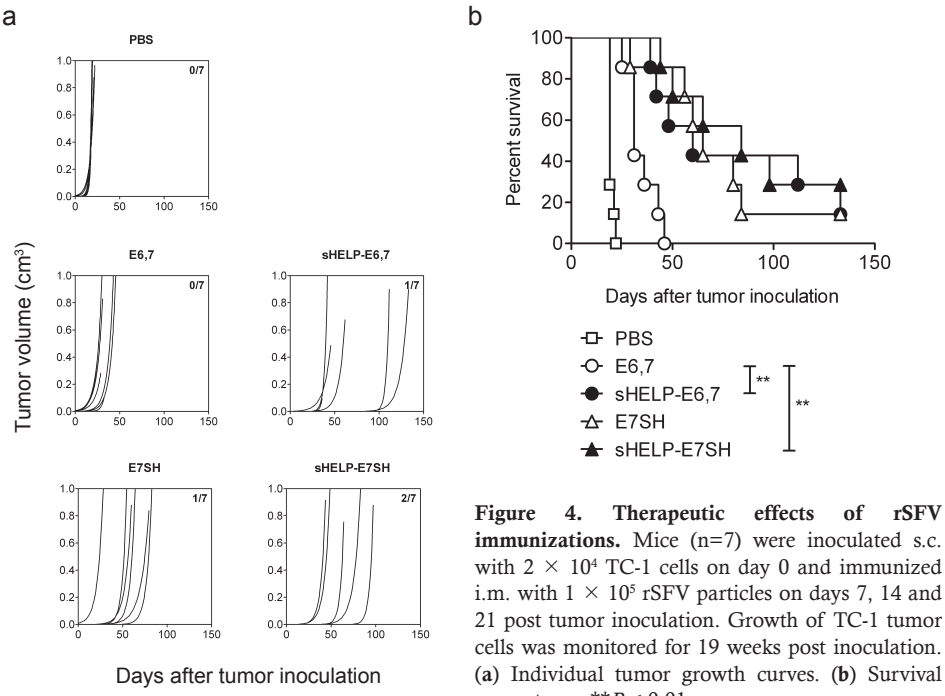

Figure 4. Therapeutic effects of rSFV immunizations. Mice (n=7) were inoculated s.c. with 2×10^4 TC-1 cells on day 0 and immunized i.m. with 1×10^5 rSFV particles on days 7, 14 and 21 post tumor inoculation. Growth of TC-1 tumor cells was monitored for 19 weeks post inoculation. (**a**) Individual tumor growth curves. (**b**) Survival percentages. $^{**}P < 0.01$.

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were inoculated. After this time-point the frequency of the E7-specific $CD8^+$ cells in blood remained constant till day 84 after the first immunization in all groups of mice (**Figure 5a, 5b**). Consistent with two immunizations at optimal dose given in a 2-week interval (**Figure 2b**), sigHELP-KDEL inclusion also increased the response of two immunizations with a 1-week interval with suboptimal doses of E7SH or E6,7 at all time points studied. The responses with suboptimal dose, as to be expected, were lower than with the optimal doses (**Figure 5b**). The E7-specific $CD8^+$ cell population in mice immunized with sigHELP-KDEL-expressing rSFV contained higher percentage of T_{EM} and T_{eff} cells in blood irrespective of the target antigens expressed by the rSFV (E6,7 or E7SH) (**Figure 5c**). Mice were monitored till week 12 post tumor inoculation. All immunizations delayed tumor growth ($P < 0.001$) (**Figure 5d and Supplementary Figure S2**) and all mice immunized with rSFV containing sigHELP-KDEL remained tumor free (Percentage of tumor free mice: E6,7: 57%; sHELP-E6,7: 100%; E7SH: 71%; sHELP-E7SH: 100%) (**Figure 5d**). Thus, immunization with rSFV induces potent memory T-cell response that effectively protects mice from the formation of TC-1 tumors.

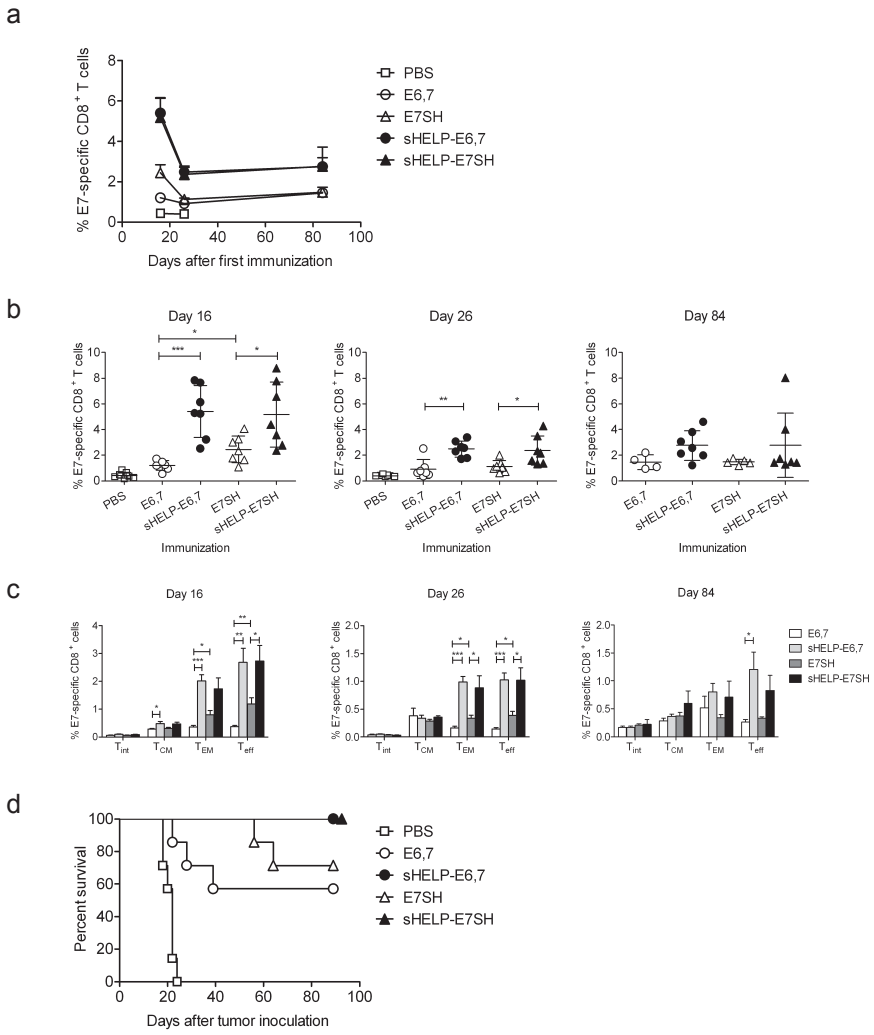

Figure 5. Memory response induced by rSFV immunizations. Mice (n=7) were immunized i.m. with 1×10^5 rSFV particles on days 0 and 7. Three week after the last immunization, mice were inoculated s.c. with 2×10^4 TC-1 cells. (**a, b**) Kinetics of the frequency of E7-specific cells in the total $CD8^+$ blood cells and (**c**) the T cell phenotypes on days 16, 26 and 84 after first immunizations (Days 9, 19 and 77 after last immunizations; Days -12, -2, 56 of TC-1 tumor cells inoculation). Percentages represent frequencies of each cell subset in E7-specific $CD44^+CD8^+$ cells. Mice were followed for 12 weeks after tumor inoculation. (**d**) Survival curves. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

DISCUSSION

In this study we determined whether the inclusion of specific CD4 helper epitopes and ER localization/retention signals in an rSFV vector further enhances the immunogenicity of rSFV-based vaccines. Inclusion of sigHELP-KDEL enhanced vaccine efficacy regardless of the antigenic nature of the HPV-target proteins (E6,7 and E7SH protein). Inclusion of TTFC only mildly enhanced the response. The

enhanced antitumor response achieved by the inclusion of sigHELP-KDEL within the rSFV vectors is most likely due to the induction of increased numbers of antigen-specific $CD8^+$ effector cells with multiple effector functions. Vaccine-induced antigen-specific $CD8^+$ cells expressed both effector and memory markers and immunization with the modified sigHELP-KDEL vaccines resulted in therapeutic and prophylactic antitumor responses even at a remarkably low dose of replicon particles, i.e., 10^5 replicon particles per immunization.

Strong cellular immunity against both E7, E6 proteins is correlated with a better prognosis of HPV-induced malignancies[10-12,29]. We synthesized rSFVs expressing carrier proteins fused with functional HPV E6 and E7 proteins. Apart from including the carrier proteins, we also generated and characterized rSFV vectors encoding a shuffled E7 protein with and without carrier proteins. Differences in immunogenicity of E6,7 and E7SH were not observed at high doses of rSFV immunizations (**Figure 2**). Interestingly, when mice were immunized with a 50-fold lower dose of rSFVs, a significant higher frequency of E7-specific $CD8^+$ T cells was induced in mice immunized with E7SH after the booster immunization (**Figure 5a, 5b**). This increase in E7-specific $CD8^+$ T cell frequency led to better protection against tumor challenge (**Figure 5d**).

Activation of Th cells is crucial for the induction and maintenance of CTL responses against antigens, certainly in case of low-immunogenicity[30]. Also in patients with cervical cancer, supportive Th immunity, as characterized by the secretion of Th1/Th2 cytokines such as IFN-γ, IL-2 and IL-5 has been shown to be protective[11,31]. Moreover, in patients with high-grade cervical intraepithelial neoplasia and carcinoma, the cytokine balance is more Th2-prone[11]. Fusion of Th epitopes and target antigens allows the simultaneous activation of both Th cells and CTLs. Bystander activated Th cells can license APCs through CD40L-CD40 interaction, resulting in efficient antigen-processing, up-regulation of both MHC and co-stimulatory molecules and to prime naïve CTLs[32]. Activated Th cells can as well directly influence expansion of activated $CD8^+$ T cells, through the secretion of pro-inflammatory cytokines such as IL-2 (ref. 33). Combined, these effects enlarge the pool of antigen-specific $CD8^+$ T cells by both enhancing clonal expansion of CTLs, reducing $CD8^+$ T cell death and memory T cell formation[30,34].

Within the rSFV vaccines tested here, only sigHELP-KDEL, but not TTFC, strongly enhanced the frequency of E7-specific CTLs relative to the standard rSFV vector. In contrast, when using HPV E7SH DNA vaccines, inclusion of TTFC also significantly increased immunogenicity relative to the parental vaccine[23]. We speculate that rSFV vectors already provide a certain level of T cell help, to which addition of the TTFC helper epitope adds little. The higher immunogenicity of sigHELP-KDEL may then be explained by the high level of protein expression by sigHELP-KDEL expressing rSFV (**Figure 1b, 1c**), potentially because of ER localization, resulting in

prolonged antigen expression in rSFV-infected cells[35,36]. In addition, increased ER-stress related apoptosis may also lead to increased exposure of antigen to APCs, thereby increasing vaccine immunogenicity[37]. And the universal helper epitope cassette present within this vaccine is likely to also increase the magnitude of the vaccine-induced T helper response.

With both the therapeutic and prophylactic rSFV treatment schedules, we observed a marked improvement of anti-tumor responses with sHELP-E6,7 immunizations compared to E6,7 immunizations. In the therapeutic immunization schedules (**Figure 4**) differences in antitumor responses between sHELP-E7SH and E7SH immunizations were not revealed, yet in the prophylactic schedules, sHELP-E7SH resulted in better antitumor responses than E7SH (**Figure 5d and Supplementary Figure S2**). Furthermore, the differences in ability to control tumor growth were positively correlated with the frequency of circulating E7-specific $CD8^+$ effector T cells. The functional characteristics of the HPV-specific T-cell responses induced by the different rSFV vaccines, as assessed by IFN-γ secretion, release of toxin granules and cytotoxic capacity were indistinguishable. Thus the high intrinsic anti-tumor and memory activity of the T cells induced with the modified vectors is not affected as a result of the enhanced expansion of the E7-specific T cell pool. This suggests that, also within the context of rSFV-based vaccines, the frequency of HPV-specific effector cells is a main determinant of tumor inhibition in the permutations studied here.

To conclude, the inclusion of a series of immunogenic Th epitopes and ER targeting signal further increases the immunogenicity of rSFV vaccines, resulting in therapeutic and prophylactic anti-tumor effects with a very low-dose of vaccine. For clinical applications these modifications in antigen design will likely also increase antitumor responses and/or allow the use of lower doses of vaccine resulting in similar immune responses compared to the unmodified vectors. We anticipate that this strategy can readily be applied to enhance immunogenicity of other viral vector platforms and/or target antigens with low immunogenicity.

MATERIALS AND METHODS

Construction of rSFV replicon vectors. pSFVeE7SH was constructed by cloning the NotI-E7SH-XmaI fragment synthesized by PCR using the plasmid DNA TTFC-E7SH[20] as a template into the PspOMI and XmaI sites of pSFVeE6,7 (ref. 13) and the E6,7 fragment from pSFVeE6,7 was removed by SmaI and PspOMI digestion. pSFVe-TTFC-E6,7 was constructed by cloning the PspOMI-TTFC-PspOMI fragment synthesized by PCR using TTFC-E7SH as a template into the PspOMI sites of pSFVeE6,7. pSFVe-TTFC-E7SH was constructed by cloning the NotI-TTFC-E7SH-PmeI synthesized by PCR using TTFC-E7SH as a template into the SmaI and PspOMI sites of pSFVeE6,7 and the E6,7 fragment from pSFVeE6,7 was removed by SmaI and PspOMI digestion. To construct pSFVe-sigHELP-E6,7-KDEL, three sequential clones were constructed. First, the plasmid DNA sigHELP-E7SH-KDEL[23] was point-mutated to remove two SpeI sites flanking the sig region to produce

^{m}sig^{m}-HELP-E7SH-KDEL using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies, Amstelveen, The Netherlands). Second, the XhoI-E6,7-KDEL-XbaI fragment, synthesized by PCR using pSFVeE6,7 as a template, was ligated to the XhoI and XbaI sites of ^{m}sig^{m}-HELP-E7SH-KDEL to produce ^{m}sig^{m}-HELP-E6,7-KDEL. The fragment KDEL was synthesized by PCR. Third, the E6,7 fragment from pSFVeE6,7 was removed by SmaI and PspOMI digestions and replaced by the NotI-^{m}sig^{m}-HELP-E6,7-KDEL-SmaI fragment which was synthesized by PCR to produce pSFVe-^{m}sig^{m}-HELP-E6,7-KDEL (pSFVe-sigHELP-E6,7-KDEL). To construct pSFVe-sigHELP-E7SH-KDEL, pSFVeE6,7 was digested with SmaI and PspOMI to remove the E6,7 fragment and ligated to the NotI-^{m}sig^{m}-HELP-E7SH-KDEL-SmaI fragment synthesized as previously described. All restriction enzymes were purchased from Thermo Scientific (Landsmeer, The Netherlands). Sequences of all constructs were confirmed by sequencing.

Production, purification and titer determination of rSFV. rSFV particle production was performed as previously described[13]. In brief, the *in vitro* transcribed RNA of the pSFV and pSFV-Helper 2 vectors were co-transfected into BHK-21 cells at a molar ratio of 1:1. Transfected BHK-21 cells were cultured at 30°C with 5% CO_2 for 48 hours to produce rSFV particles. The supernatant containing rSFV particles was collected and purified on a discontinuous sucrose density gradient. Purified rSFV particles were serially diluted and titrated with BHK-21 cells. rSFV particles were stored at -80°C.

Cell culture. Baby hamster kidney cells (BHK-21) were obtained from the American Type Culture Collection (No. CCL-10) and cultured in RPMI1640 (Life technologies, Bleiswijk, The Netherlands) with 10% FBS (Lonza, Basel, Switzerland). C3 cells and TC-1 cells were cultured in IMDM (Life technologies) with 10% FBS, which for TC-1 cultures was supplemented with 1 mM MEM sodium pyruvate, and 1× MEM non-essential amino acids (both from Life technologies) and 30 μM β-mercaptoethanol. All cells were cultured in the presence of 100 U/ml penicillin and 100 μg/ml streptomycin (Life technologies) at 37°C with 5% CO_2.

Western blot analysis. BHK-21 cells (5×10^5 cells) were incubated with rSFV (5×10^6 particles) for 24 hours. Cells were lysed on ice in TENT-SDS lysis buffer (1% SDS, 50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, and 0.5% Triton-X-100, pH7.5) supplemented with 0.2 mM phenyl-methane-sulphonyl-fluoride. Cell lysates were spun down and the supernatants were harvest and analyzed by SDS/PAGE and western blotting. HPV E7 protein was detected with a monoclonal mouse anti-HPV E7 antibody (Ab) (1/5000, Zymed Lab, South San Franciscom, CA, USA). Alkaline phosphatase-conjugated goat anti-mouse IgG Ab (Southern Biotech, Alabama, USA) was used as a secondary antibody. The E7 proteins were visualized by colorimetric detection using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate substrates solution (Roche applied science, Almere, The Netherlands). Densitometry analysis was performed using ImageJ software (NIH software).

Protein expression by pulse labeling. BHK-21 cells (5×10^5 cells) were incubated with 5×10^6 rSFV replicons. Six hours after incubation, supernatant was removed and the cells were washed with phosphate buffered saline (PBS) 3 times. The cells were further cultured in L-methionine and L-cysteine-free DMEM for 30 min following addition of [^{35}S]-protein labeling mix (0.37 Mbq/well) (PerkinElmer, Groningen, The Netherlands). After 1 hour of [^{35}S]-methionine labeling, cells were washed three times with PBS and cultured in medium

supplemented with 5 mM of methionine and cysteine. After 1, 6, 18, 24 or 42 hours of [^{35}S]-methionine labeling, cells were washed with cold PBS and lysed with TENT-SDS lysis buffer (1% SDS, 50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, and 0.5% Triton-X-100, pH7.5) containing 0.2 mM phenyl-methane-sulphonyl-fluoride. Cell lysates were analyzed by SDS/PAGE and autoradiography.

Mice. C57BL/6JOlaHsd female inbred mice of 8 to 10 weeks old were obtained from Harlan CPB (Zeist, The Netherlands). Housing and animal experiments (DEC: 6405) were approved by the local Animal Experimentation Ethical Committee (the Institutional Animal Care and Use Committee of the University Medical Center of Groningen).

rSFV immunizations. Mice were prime- and booster immunized intramuscularly (i.m.) with 50 µl of 5 × 10^6 rSFV particles (25 µl/thigh muscle). For negative controls, the same volume of PBS was injected i.m.. To determine the effect of immunizations against tumor growth, mice were immunized with a suboptimal dose of rSFV particles (1 × 10^5 particle/mouse).

Detection of HPV E7-specific CD8$^+$ cells. Mouse peripheral blood was collected by orbital puncture and erythrocytes were lysed. Cells were stained with PE-conjugated H-2D^b $E7_{49-57}$ tetramers for 10 min at room temperature. Followed by staining with PE-Cy7-anti-CD8a Ab (clone: 53-6.7), PerCP-Cyanine5.5-anti-CD44 Ab (clone: IM7), APC-anti-CD62L Ab (clone: MEL-14) and eFluor 450-anti-CD127 Ab (clone: A7R34) antibodies for 20 min at 4°C. Antibodies were purchased from eBioscience (Vienna, Austria). Cells were stained with 4'-6'-diamidino-2-phenylindole to exclude dead cells before analyzing with LSR-II flow cytometer (BD Bioscience, Breda, The Netherlands). Data were analyzed using Flowjo software (Tree Star).

Detection of IFN-γ and degranulation of HPV E7-specific CD8$^+$ cells. Splenocytes isolated from immunized mice were stimulated with 10 µg/ml of E7 synthetic peptides (RAHYNIVTF, H-2D^b) in the presence of 1 µg/ml anti-CD28 Ab (clone: PV-1, Bioceros B.V., Utrecht, The Netherlands), eFluor 660-anti-CD107a Ab (clone: eBio1D4B) and eFluor 660-anti-CD107b Ab (clone: eBioABL-93) in a 96-well plate at 37°C with 5% CO_2. Brefeldin A (1 mg/ml) was added 1 hour after culture. Cells were further incubated for 4 hours. Cells were then harvested, washed and stained with LIVE/DEAD fixable violet dead cell stain kit (Life technologies). Followed with PE-Cy7-anti-CD8a (clone: 53-6.7) staining at 4°C for 20 min. Cells were then fixed in 4% paraformaldehyde, permeabilized with Perm/wash buffer (BD Bioscience) and stained with PerCP-Cyanine5.5-anti-IFN-γ Ab (clone: XMG1.2) at 4°C for 30 min. Antibodies were purchased from eBioscience; FACS analysis was conducted with LSR-II flow cytometer, and data were analyzed using Flowjo software (Tree Star).

Bulk CTL assay. Stimulator TC-1 cells were cultured in the presence of 50 U/ml recombinant murine IFN-γ (Peprotech, London, UK) for 48 hours. The TC-1 cells were then irradiated (100 Gy) and co-cultured with effector cells, splenocytes, at a ratio of 1:25 in a T25 flask at 37°C with 5% CO_2. Recombinant human IL-2 (4 U/ml) (Peprotech, London, UK) was added on day 5 of the culture. After 7 days culture, splenocytes were harvested and co-cultured with ^{51}Chromium (^{51}Cr)-labeled C3 target cells. C3 cells were cultured in the presence of recombinant murine IFN-γ (50 U/ml) 48 hours before co-culture with effector cells. C3 cells were labeled with ^{51}Cr (100 µCi/2 × 10^6 cells) (PerkinElmer, Groningen, The Netherlands) for 1 hour at 37°C.

Co-culture of effector cells and target cells were performed in 96 well plate at 4 E:T ratios in triplicates at 37°C with 5% CO_2 for 4 hours. ^{51}Cr in the cells supernatants was measured with a RiaStar manual gamma counter (Packard, Meriden, CT). The percentage of cytotoxicity was calculated with the formula % specific release = ((experimental release – spontaneous release) / (maximal release – spontaneous release)) count per minute (c.p.m).

Tumor inoculation. Mice were inoculated subcutaneously (s.c.) with 2×10^4 TC-1 cells suspended in 0.2 ml PBS. Tumor volume was measured by caliper. The volumes of cylinder-shaped tumors were calculated with the formula $0.7854 \times \text{width}^2 \times \text{length}$ (cm^3). Volumes of round tumors were calculated with the formula $0.5236 \times \text{diameter}^3$ (cm^3). Mice were euthanized when the tumor volume reached 1 cm^3.

Statistical analysis. Data were analyzed with GraphPad Prism software (La Jolla, CA). Differences between two groups were calculated with the Mann-Whitney *U* test. Differences between two survival curves were calculated using the log-rank (Mantel-Cox) test. $P < 0.05$ was considered as statistically significant.

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CONFLICT OF INTEREST

Toos Daemen and Hans W. Nijman are co-founders of ViciniVax, a spin-off company from the University Medical Center Groningen developing therapeutic cancer vaccines. Ton N. Schumacher, John B. Haanen and Koen Oosterhuis are inventors on a patent application covering vaccine formats and are as such entitled to a fraction of possible future royalty income, as per NKI-AVL regulations.

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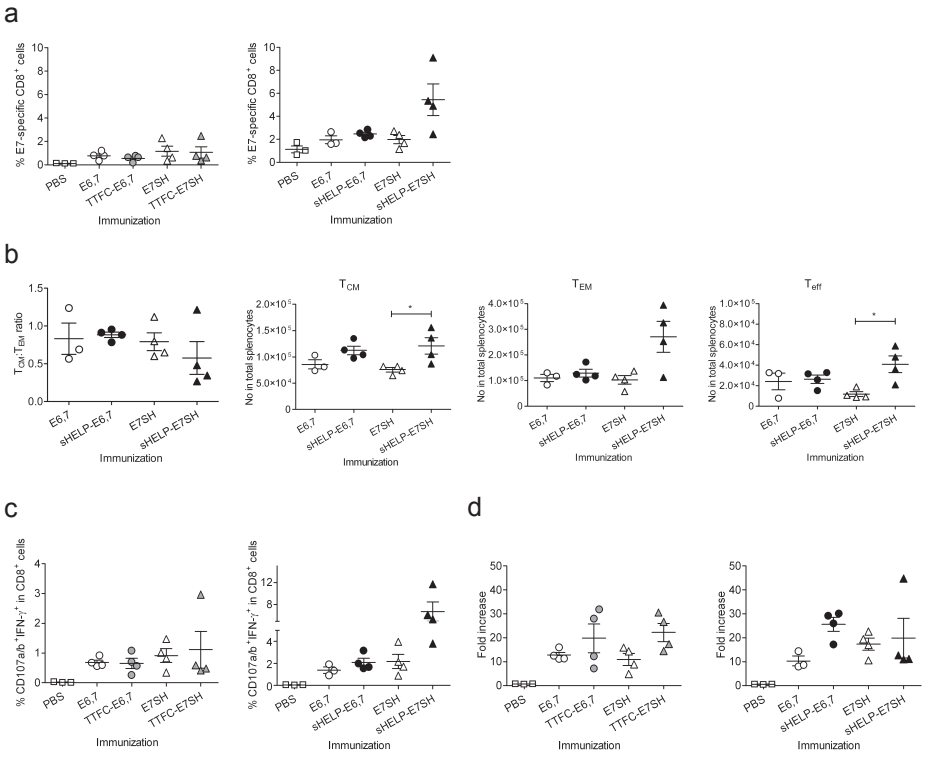
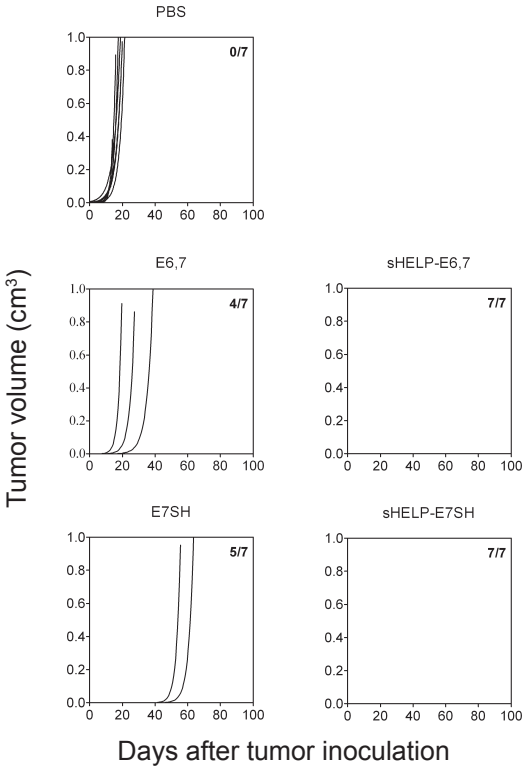
SUPPLEMENTARY MATERIAL



Supplementary Figure S1. Phenotypes and function of E7-specific CD8$^+$ cells post recombinant Semliki Forest virus (rSFV) prime-boost immunizations. Mice were primed and boosted intramuscularly with 5 $\times 10^6$ rSFV particles with a 2-week interval. Ten days after the last immunization, mice were sacrificed and splenocytes were isolated for phenotypic analysis. (**a**) Percentage of E7-specific cells in CD8$^+$ spleen cells. (**b**) T_{CM} to T_{EM} ratio, absolute number of T_{CM} (CD62L$^+$CD127$^+$), T_{EM} (CD62L$^-$CD127$^+$) and T_{eff} (CD62L$^-$CD127$^-$) and in total E7-specific CD8$^+$CD44$^+$ spleen cells. (**c**) Splenocytes were stimulated with $E7_{49\text{-}57}$ peptides (RAHYNIVTF, H-2D^b) in the presence of BFA at 37°C. After 4 hours of stimulation, cells were harvested, subjected to intracellular cytokine staining and analyzed by flow cytometry. (**d**) Fold increase of the frequency of E7-specific cells in CD8$^+$ spleen cells after 7-day re-stimulation with irradiated TC-1 cells. The fold increase of E7-specific CD8$^+$ cells was calculated by dividing the frequency of E7-specific CD8$^+$ cells on day 7 after re-stimulation over the frequency of E7-specific CD8$^+$ cells on day 0 after re-stimulation. Data represent mean ± SEM of each group (n = 3-4). * $P < 0.05$.



Supplementary Figure S2. Effect of prophylactic recombinant Semliki Forest virus (rSFV) immunizations on tumor growth. Mice (n=7) were immunized intramuscularly with 1×10^5 rSFV particles on days 0 and 7. Three week after the last immunization, mice were inoculated subcutaneously with 2×10^4 TC-1 cells. Individual tumor growth curves are presented.

5

Effect of an Immunogenic Carrier Protein on the Efficacy of an Alphavirus-based Hepatitis C Virus Vaccine

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Study in progress

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Effect of an Immunogenic Carrier Protein on the Efficacy of an Alphavirus-based Hepatitis C Virus Vaccine

ABSTRACT

We previously developed therapeutic vaccines against hepatitis C virus (HCV) infections. These vaccines are based on recombinant Semliki Forest virus (rSFV) replicon particles and encode all nonstructural proteins of HCV or parts of them. We also demonstrated that a highly immunogenic carrier protein that consists of a series of helper T cell (Th) epitope and endoplasmic reticulum (ER) targeting signals (sigHELP-KDEL) increases the efficacy of an rSFV-based vaccine against human papillomavirus (HPV). To investigate whether the inclusion of sigHELP-KDEL can augment HCV-specific immune responses, we applied this antigen design also for one of our candidate HCV vaccines.

An rSFV vector expressing a fusion protein of sigHELP-KDEL and the nonstructural proteins NS3/4A of HCV (rSFVe-sHELP-NS3/4A) was generated and its immunogenicity was compared to the parental vaccine, rSFVeNS3/4A. Similar to the effect observed with the HPV vaccines, insertion of sigHELP-KDEL resulted in higher protein synthesis. Yet, whereas the immune response elicited by the modified HPV vaccine was enhanced by insertion of sigHELP-KDEL, rSFVe-sHELP-NS3/4A and rSFVeNS3/4A immunizations were as efficacious. Both vaccines induced similar frequencies and activities of functional HCV-specific $CD8^+$ T cells. Also the frequencies of effector and memory population were identical.

To conclude, inclusion of the Th epitopes and ER targeting signals into rSFV expressing HCV NS3/4A does not alter the $CD8^+$ T-cell response compared to that induced by the parental rSFVeNS3/4A vaccine. This implies that insertion of sigHELP-KDEL cannot be considered a universal strategy to augment rSFV vaccine efficacy. Effects of carrier proteins on vaccine efficacy thus also depend on the target antigen expressed by the vaccine.

INTRODUCTION

Antigen-specific T-cell responses are essential to resolve acute and chronic hepatitis C virus (HCV) infections[1-3]. Recently developed novel HCV-specific direct acting antiviral (DAA) offer the prospect of interferon-free treatments with an increased curative rate in patients infected with HCV genotype 1. However, treatment with DAA is sometimes associated with multiple side effects, drug-drug interaction, development of resistance-associated variants, and importantly DAAs do not induce protective immunity in patients[4]. Aiming to induce HCV-specific immunity we developed therapeutic HCV vaccines based on a recombinant Semliki Forest virus (rSFV) vector. These vaccines aim to induce cellular immune responses against HCV infected cells expressing the nonstructural (NS) proteins of HCV. The NS proteins are genetically conserved and immunogenic. We showed that these rSFV-based vaccines result in the induction of functional $CD8^+$ T cells containing both effector and memory population and delayed growth of HCV NS protein-expressing tumor *in vivo*[5].

Helper T (Th) cells are indispensable for the induction and maintenance of memory $CD8^+$ T-cell responses[6]. Oosterhuis *et al* demonstrated that the expression of a series of Th epitopes in a DNA-based vaccine stimulated both Th cells and cytotoxic T lymphocytes (CTLs). They furthermore demonstrated that inclusion of endoplasmic reticulum (ER) targeting signals within these DNA vaccines increased antigen stability[7]. The combined modifications strongly enhanced the immunogenicity of their DNA vaccine.

We previously demonstrated that the inclusion of Th and ER targeting signals also strongly enhanced vaccine immunogenicity of an rSFV vaccine expressing human papillomavirus (HPV) early proteins (**Ip PP *et al.*, submitted, Chapter 4**). Immunizations with these novel vaccines resulted in the induction of a higher frequency of functional antigen-specific T cells and anti-tumor activity already at a very low vaccine dosage. Thus these modifications may be applied universally to up-regulate the immunogenicity of rSFV-based vaccine.

In the present study, aiming to enhance the immunogenicity of the rSFV-based HCV vaccine, we constructed a novel rSFV-based HCV vaccine expressing Th epitopes, ER targeting signals and HCV NS3/4A proteins. The new vaccine was characterized *in vitro* and the *in vivo* efficacy was compared to the parental rSFV vaccine expressing HCV NS3/4A.

RESULTS AND DISCUSSION

We modified our previously developed SFV replicon-based HCV vaccine by inserting a series of Th epitopes, including the TTFC P30 pan DP epitope, PADRE pan DR epitope and HIV NEF pan DQ epitope (HELP)[7]. In addition, a human growth hormone signal peptide (sig)[8] and a KDEL sequence[9,10] were inserted to enable

localization and retention of the antigen within the ER. pSFVe-sigHELP-NS3/4A-KDEL was thus constructed (**Figure 1a**) and the corresponding rSFV particles were produced as described in the materials and methods section. To characterize the rSFVe-sigHELP-NS3/4A-KDEL (rSFVe-sHELP-NS3/4A) and rSFVeNS3/4A vaccine, BHK-21 cells were infected with rSFV replicon particles and cell lysates were harvested 24 hours after incubation. Infection with rSFVe-sHELP-NS3/4A or rSFVeNS3/4A resulted in expression of the sigHELP-NS3/4A-KDEL fusion protein (95 kDa, lane 2, upper band) and NS3/4A protein (81 kDa, lane 3, upper band), respectively (**Figure 1b**). A protein of a smaller size (sigHELP-NS3: 86 kDa, NS3: 75 kDa), which is generated upon cleavage between NS3 and NS4A by the HCV NS3 protease, was observed in cell lysate infected with either rSFV particles.

We previously demonstrated that inclusion of sigHELP-KDEL in HPV-expressing rSFV enhanced production and stability of HPV proteins (**Ip PP *et al.*, submitted, Chapter 4**). BHK-21 cells infected with rSFVe-sHELP-NS3/4A similarly produced a higher amount of transgene compared to cells infected with rSFVeNS3/4A as demonstrated by a one-hour pulse labeling of protein using [^{35}S]-methionine/cysteine. However in contrast to the HPV proteins, sigHELP-KDEL did

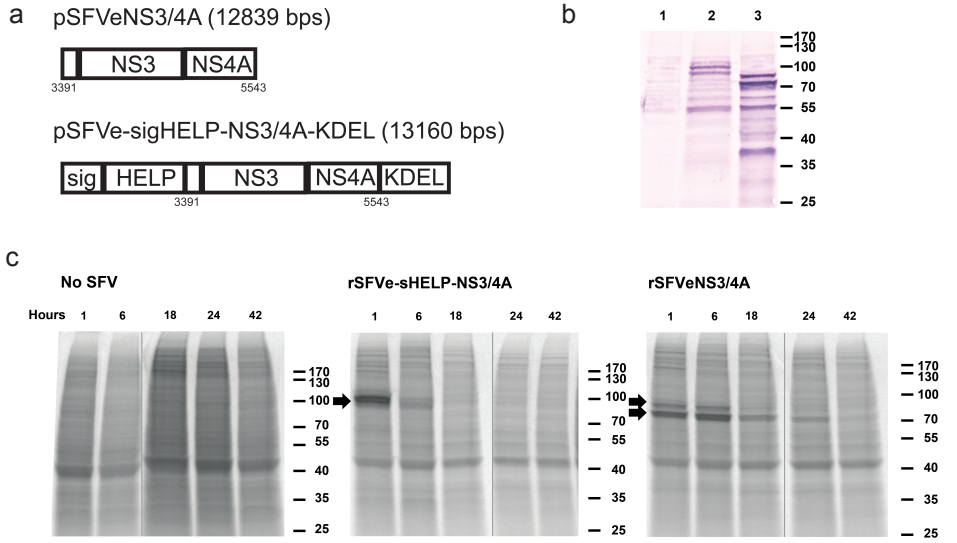

Figure 1. Expression and stability of HCV NS3/4A protein in BHK-21 cell upon rSFVeNS3/4A or rSFVe-sHELP-NS3/4A infection. (**a**) Schematic representation of the plasmid SFV DNA, the number indicates the nucleotide position in the plasmid DNA containing the full genome of HCV genotype 1a (H/FL). (**b**) BHK-21 cells were infected with rSFV particles at a multiplicity of infection of 10 for 24 hours and cell lysates were collected for western blot analysis. HCV NS3 protein was detected using goat anti-NS3 antibodies. Lane 1: no rSFV; lane 2: rSFVe-sHELP-NS3/4A; lane 3: rSFVeNS3/4A. (**c**) Stability of protein was analyzed by [^{35}S]-methionine/cysteine pulse-chase labeling. BHK-21 cells were incubated with rSFVe-sHELP-NS3/4A, rSFVeNS3/4A or medium (no rSFV). Cells were pulsed with [^{35}S]-methionine/cysteine for 1 hour at 6 hours after addition of rSFV particles. Cell lysates were collected at 1, 6, 18, 24 or 42 hours after [^{35}S]-labeling. Different gels that were run at the same time are separated with vertical lines.

not increase stability of the NS3/4A protein but rather decreased its stability. The sHELP-NS3/4A-KDEL protein was only detectable till 6 hours after [^{35}S]-labeling while NS3/4A protein was detectable up to 24 hours (**Figure 1c**).

Next, we determined the immunogenicity of both vaccines *in vivo*. Mice were immunized intramuscularly on day 0 and day 14 with 5 × 10^6 purified rSFV particles and the frequency of NS3-specific CD8$^+$ T cells in peripheral blood was determined on days 7, 11, 18, 21 and 24 after immunization (**Figure 2a**). The frequency of NS3-specific CD8$^+$ T cells in mice immunized with rSFVe-sHELPNS3/4A (sHELP-NS3/4A) at all time points tested was as high as the frequency observed after rSFVeNS3/4A (NS3/4A) immunizations. On day 24, all mice were sacrificed, splenocytes were isolated and the cells characterized. Both rSFV vaccines induced equal numbers of NS3-specific CD8$^+$ T cells composed of equal frequencies of effector T cells, effector memory T cells and central memory T cells (**Figure 2b, 2c**).

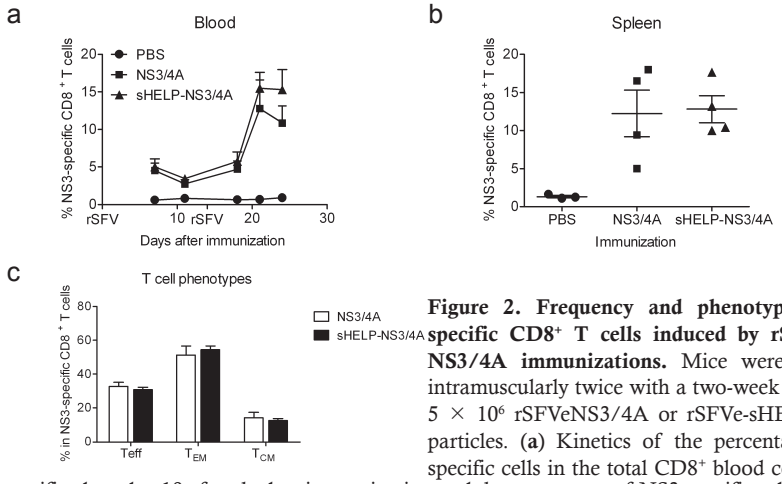

Figure 2. Frequency and phenotypes of NS3-specific CD8$^+$ T cells induced by rSFV-sHELP-NS3/4A immunizations. Mice were immunized intramuscularly twice with a two-week interval with 5 × 10^6 rSFVeNS3/4A or rSFVe-sHELP-NS3/4A particles. (**a**) Kinetics of the percentage of NS3-specific cells in the total CD8$^+$ blood cells. (**b**) Mice were sacrificed on day 10 after the last immunization and the percentage of NS3-specific cells in the total CD8$^+$ splenocytes was determined. (**c**) Surface markers for the NS3-specific CD8$^+$ T cell subsets: effector T cells (T_{eff}), CD44$^+$CD62L$^-$CD127$^-$, effector memory T cells (T_{EM}), CD44$^+$CD62L$^-$CD127$^+$, central memory T cells (T_{CM}): CD44$^+$CD62L$^+$CD127$^+$. Percentages of each subset in the total NS3-specific CD44$^+$CD8$^+$ spleen cells are shown. Data represent mean ± or + S.E.M (n=3-4).

Next, the activity of HCV-specific T cells upon antigen re-stimulation was measured using a CFSE dilution assay. Splenocytes from control or immunized mice were labeled with CFSE and cultured in the presence or absence of Hepa1-6^V-nsPs cells for 4 days to induce proliferation of HCV-specific T cells. Hepa1-6^V-nsPs cells are murine hepatoma cells expressing all NS proteins of HCV[5]. Antigen-specific CD8$^+$ T cells from rSFV-immunized mice but not from control mice had an increased level of proliferation upon HCV-specific re-stimulation (**Figure 3**, black bars). In the absence of Hepa1-6^V-nsPs cells, the proliferation of CD8$^+$ T cells isolated from immunized mice was higher compared to the background level observed in control splenocytes, suggestive of strong *in vivo* activation of T cells (**Figure 3,** white bars). Both rSFV

vaccines induced similar $CD8^+$ T-cell response. A further analysis of the activation of $CD4^+$ T cells is needed to demonstrate if immunization with rSFVe-sHELP-NS3/4A actually results in a stronger $CD4^+$ T-cell response as a consequence of the inclusion of Th epitopes.

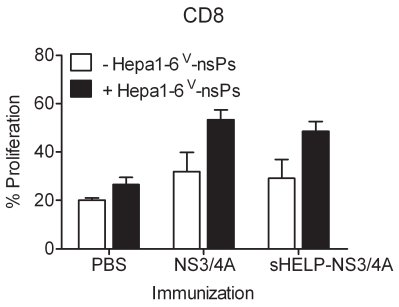

Figure 3. Proliferation of HCV-specific T cells upon *in vitro* re-stimulation. Splenocytes isolated from mice immunized twice with rSFV as described in figure 2 were labeled with CFSE and cultured in the presence or absence of irradiated Hepa1-6^V-nsPs cells at a 25:1 ratio. After 4 days of culture, cells were harvested, stained with anti-CD8a antibodies and analyzed using flow cytometer. Percentages of proliferation represent the percentage of proliferating daughter $CFSE^+CD8^+$ cells (sum of generation 1 to 7) in the total $CFSE^+CD8^+$ cell population (sum of generation 0 to 7). Data represent mean + S.E.M (n=3-4).

5

We previously demonstrated that the insertion of sigHELP and KDEL resulted in a 2- to 4-fold increased expansion of antigen-specific cells in rSFV-based HPV vaccines dependent of the antigen expressed (HPV E6,7 or shuffled E7 protein (E7SH)) (**Ip PP *et al.*, submitted, Chapter 4**). At high dose rSFV immunization (5×10^6 particle/mouse), mice immunized with rSFVe-sHELP-E7SH had an even higher number of E7-specific cells compared to low dose immunization. This enhanced effect of sigHELP-KDEL was not seen with the vaccine expressing the E6,7 fusion protein of HPV.

rSFVeNS3/4A immunizations resulted in a very high frequency of NS3-specific CD8 T cells (13% on day 7 after the booster immunization) (**Figure 2a**). Thus, the NS3-specific T-cell proliferative response may have reached already such a high level that insertion of sigHELP-KDEL cannot further up-regulate the response. We also observed that insertion of sigHELP-KDEL into the HCV vaccine did not increase the stability of the NS3/4A protein. We previously demonstrated that the efficacy of rSFV-based HPV vaccines is influenced by the stability of the transgene expressed. This could be yet another explanation on why immunization with rSFVe-sHELP-NS3/4A is just as efficacious as rSFVeNS3/4A immunization. To investigate the contribution of sigHELP-KDEL in this rSFV vaccine, lower dose immunization may be needed.

In the present study, we focused on the T-cell response against one immunodominant NS3 epitope ($NS3_{603\text{-}611}$) that is a known strong binder for MHC class I H-$2D^b$ molecule and is generally detected as a result of other immunization approaches[11]. Induction of robust T-cell response against the $NS3_{603\text{-}611}$ peptide might be due to the presence of Th epitopes located at the 5' end of the CTL epitopes, as predicted in the Immune Epitope Database and analysis resource (IEDB) (**Table**

a

Protein/position	Sequence	MHC class II prediction IEDB- H-2-IA^b (**<10 strong binding**) 5' of CTL epitope	complete CTL epitope	3' of CTL epitope
HCV NS3$_{603-611}$	GAVQNEVTL	**9,35** - 55,70	18,51 - 32,13	16,70 - 51,34
HPV E7$_{49-57}$	RAHYNIVTF	33,38 - 53,38	56,31 - 79,26	69,56 - 86,81
OVA$_{257-264}$	SIINFEKL	51,45 - 69,97	51,88 - 81,68	**9,57** - 82,66

b

HCV NS3$_{603-611}$ Start	End	Peptide	Percentile_rank	HPV E7$_{49-57}$ Start	End	Peptide	Percentile_rank	OVA$_{257-264}$ Start	End	Peptide	Percentile_rank
586	600	IRLKPTLHGPTPLLY	9,46	243	257	SEEEDEIDGPAGQAE	45,50	239	253	MSMLVLLPDEVSGLE	**51,45**
587	601	RLKPTLHGPTPLLYR	**9,35**	244	258	EEEDEIDGPAGQAEP	40,53	240	254	SMLVLLPDEVSGLEQ	57,90
588	602	LKPTLHGPTPLLYRL	9,53	245	259	EEDEIDGPAGQAEPD	34,31	241	255	MLVLLPDEVSGLEQL	58,88
589	603	KPTLHGPTPLLYRL**G**	15,00	246	260	EDEIDGPAGQAEPD**R**	34,54	242	256	LVLLPDEVSGLEQLE	66,91
590	604	PTLHGPTPLLYRL**GA**	16,91	247	261	DEIDGPAGQAEPD**RA**	**33,38**	243	257	VLLPDEVSGLEQLE**S**	69,64
591	605	TLHGPTPLLYRL**GAV**	35,41	248	262	EIDGPAGQAEPD**RAH**	36,55	244	258	LLPDEVSGLEQLE**SI**	**69,97**
592	606	LHGPTPLLYRL**GAVQ**	37,59	249	263	IDGPAGQAEPD**RAHY**	41,11	245	259	LPDEVSGLEQLE**SII**	69,05
593	607	HGPTPLLYRL**GAVQN**	**55,70**	250	264	DGPAGQAEPD**RAHYN**	43,28	246	260	PDEVSGLEQLE**SIIN**	58,72
594	608	GPTPLLYRL**GAVQNE**	31,54	251	265	GPAGQAEPD**RAHYNI**	45,39	247	261	DEVSGLEQLE**SIINF**	58,70
595	609	PTPLLYRL**GAVQNEV**	18,75	252	266	PAGQAEPD**RAHYNIV**	50,11	248	262	EVSGLEQLE**SIINFE**	56,29
596	610	TPLLYRL**GAVQNEVT**	18,04	253	267	AGQAEPD**RAHYNIVT**	**53,38**	249	263	VSGLEQLE**SIINFEK**	57,34
597	611	PLLYRL**GAVQNEVTL**	**18,51**	254	268	GQAEPD**RAHYNIVTF**	**56,31**	250	264	SGLEQLE**SIINFEKL**	62,04
598	612	LLYRL**GAVQNEVTLT**	20,16	255	269	QAEPD**RAHYNIVTFC**	62,70	251	265	GLEQLE**SIINFEKLT**	66,94
599	613	LYRL**GAVQNEVTLTH**	25,75	256	270	AEPD**RAHYNIVTFCC**	62,78	252	266	LEQLE**SIINFEKLTE**	70,93
600	614	YRL**GAVQNEVTLTHP**	**32,13**	257	271	EPD**RAHYNIVTFCCK**	72,33	253	267	EQLE**SIINFEKLTEW**	**81,68**
601	615	RL**GAVQNEVTLTHPI**	24,94	258	272	PD**RAHYNIVTFCCKC**	74,42	254	268	QLE**SIINFEKLTEWT**	81,00
602	616	L**GAVQNEVTLTHPIT**	26,14	259	273	D**RAHYNIVTFCCKCD**	75,44	255	269	LE**SIINFEKLTEWTS**	53,47
603	617	**GAVQNEVTLTHPITK**	19,76	260	274	**RAHYNIVTFCCKCDS**	**79,26**	256	270	**ESIINFEKLTEWTSS**	53,51
604	618	**AVQNEVTL**THPITKY	18,71	261	275	**AHYNIVTF**CCKCDST	81,61	257	271	**SIINFEKL**TEWTSSN	**51,88**
605	619	**VQNEVTL**THPITKYI	**16,70**	262	276	**HYNIVTF**CCKCDSTL	82,43	258	272	**IINFEKL**TEWTSSNV	46,01
606	620	**QNEVTL**THPITKYIM	20,28	263	277	**YNIVTF**CCKCDSTLR	82,92	259	273	**INFEKL**TEWTSSNVM	12,94
607	621	**NEVTL**THPITKYIMT	21,71	264	278	**NIVTF**CCKCDSTLRL	83,88	260	274	**NFEKL**TEWTSSNVME	12,73
608	622	**EVTL**THPITKYIMTC	33,01	265	279	**IVTF**CCKCDSTLRLC	83,84	261	275	**FEKL**TEWTSSNVMEE	**9,57**
609	623	**VTL**THPITKYIMTCM	37,85	266	280	**VTF**CCKCDSTLRLCV	85,52	262	276	**EKL**TEWTSSNVMEER	9,77
610	624	**TL**THPITKYIMTCMS	**51,34**	267	281	**TF**CCKCDSTLRLCVQ	86,56	263	277	**KL**TEWTSSNVMEERK	10,15
611	625	L**T**HPITKYIMTCMSA	46,32	268	282	**FC**CKCDSTLRLCVQS	86,59	264	278	L**T**EWTSSNVMEERKI	16,84
612	626	THPITKYIMTCMSAD	22,86	269	283	CCKCDSTLRLCVQST	**86,81**	265	279	TEWTSSNVMEERKIK	19,59
613	627	HPITKYIMTCMSADL	23,05	270	284	CKCDSTLRLCVQSTH	85,77	266	280	EWTSSNVMEERKIKV	46,91
614	628	PITKYIMTCMSADLE	23,32	271	285	KCDSTLRLCVQSTHV	**69,56**	267	281	WTSSNVMEERKIKVY	51,01
								268	282	TSSNVMEERKIKVYL	**82,66**

Table 1. Prediction of Th epitopes at close proximity of the CTL epitope. Prediction of H-2-IA^b Th epitopes was performed using IEDB. Peptides were divided into 3 groups: peptides that contain part of the CTL epitope either at the 5' end or 3' end and peptides that contain the complete CTL epitope. (**a**) The range of percentile of the analyzed peptides. (**b**) Individual percentile of each analyzed peptide. Percentiles below 10 are considered as strong MHC class II binder. The highest and the lowest percentiles and are bolded and high-lined in grey. Peptides that contain the complete CTL epitope are also high-lined in grey. HCV NS3$_{603-611}$ (HCV genotype 1 (H77), NCBI reference sequence: NC_004102.1), OVA$_{257-264}$ (GenBank: AAB59956.1) and HPV type 16 E7$_{49-57}$ (GenBank: AAL96657.1).

1). $OVA_{265-280}$, a known Th epitope located at the 3' terminal of the CTL epitope ($OVA_{257-264}$) was also predicted by IEDB[12]. Moreover, several immunodominant tumor and viral antigens contain Th and CTL epitopes at close proximity. This co-localization of Th and CTL epitopes seems positively correlated to the dominancy of the specific CTL epitope[13,14]. So far, based on knowledge and experimental proof, no unequivocal explanations exist for this positive correlation. Nonetheless, based on this observation, one could hypothesize that the close proximity of a Th and CTL epitope in HCV NS3 supports such a potent Th response that external Th epitopes do not further augment the immune response. This hypothesis is supported by the observation that insertion of external helper epitopes to a vector expressing the HPV $E7_{49-57}$ CTL epitope, an epitope that according to IEDB is not in close proximity to a Th epitope(s), does enhance the vaccine immunogenicity.

Is the vaccine-induced immune response protective? Despite the fact that rSFVeNS3/4A immunizations induced a very high frequency of NS3-specific $CD8^+$ T cells, the immunized mice were not completely protected against the tumor growth of an HCV NS3/4A-expressing EL4 tumor[5]. Nevertheless, immunization with rSFVeNS3/4A resulted in enhanced immune pressure on the fast-growing EL4 tumor cells leading to a significant reduction of HCV nsPs expression. This resulted in delayed tumor growth and indicates that $CD8^+$ T-cell response against $NS3_{603-611}$ is protective in C57BL/6 mice. Since responses against multiple epitopes in humans have been positively correlated to a better prognosis[1,2,15,16], one may assume that responses against other protective subdominant epitopes may also play a role in tumor clearance. Further experiments are required to investigate whether the inclusion of sigHELP-KDEL could induce CTL response against subdominant epitopes.

To summarize, at a high dose rSFVeNS3/4A and rSFVe-sHELP-NS3/4A immunizations induced equal levels of NS3-specific $CD8^+$ T cells with functional effector and memory phenotypes. This result contrasts with our previous finding showing that insertion of sigHELP-KDEL in rSFV can enhance CTL responses (**Ip PP *et al.*, submitted, Chapter 4**). This contrast may be attributed to the different antigenic nature of the antigens expressed, for example by the presence of internal Th epitopes and the stability of the protein. Moreover, fusion of sigHELP-KDEL with other antigens such as hepatitis B virus core protein in a DNA vaccine also does not enhance vaccine immunogenicity[17]. Further evaluations on the possible role of sig-HELP-KDEL on rSFVeNS3/4A are required. This will include investigation of the effect of low dose rSFV immunization on the activation of $CD4^+$ T cells, the diversity and strength of $CD8^+$ T-cell responses, and their anti-tumoral effect.

MATERIALS AND METHODS

Construction of SFV plasmid and production of recombinant SFV particles. Construction of pSFVeNS3/4A (12839 bps) is previously described[5]. To generate the pSFVe-sigHELP-NS3/4A-KDEL construct, a series of Th epitopes (HELP), ER localization signal (sig),

HCV NS3/4A and ER retention signal (KDEL) were cloned into a SFV vector[5]. The BssHII-sigHELP-NotI fragment was amplified by PCR using the pVAX1-SigHELP-E7SHKDEL vector[7] (provided by K. Oosterhuis, J. B. Haanen and T. N. Schumacher, Netherlands Cancer Institute, Amsterdam, The Netherlands) as a template and ligated into pSFV4.2e to generate pSFVe-sigHELP. pSFVe-sigHELP was then linearized with NotI digestion and ligated with the NotI-NS3/4A-KDEL-NotI, which was amplified by PCR from the plasmid DNA containing the full-length cDNA of HCV H77 genotype 1a consensus sequences (H/FL) (kindly provided by Rice, CM via Apath, LLC (AIDS Research and Reference Reagent Program, Division of AIDs, NIAID, NIAID, NIH: p90HCVconsensuslongpU)[19], and the four amino acid KDEL sequence was synthesized by PCR, to produce pSFVe-sigHELP-NS3/4A-KDEL (13160 bps). Recombinant SFV particles, rSFVeNS3/4A and rSFVe-sigHELP-NS3/4A-KDEL (abbr. rSFVe-sHELP-NS3/4A) were produced in BHK-21 cells and purified with discontinuous sucrose gradient as previously described[5,18].

Cell lines. Baby hamster kidney cells (BHK-21) obtained from ATCC (No. CCL-10), were cultured in RPMI medium (Life Technologies, Bleiswijk, The Netherlands), containing 10% FCS (Lonza, Basel, Switzerland) and 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies). Hepa1-6^{V}-nsPs cells[5] were cultured in DMEM medium (GE Healthcare, Fairfield, CT, USA) containing 10% FCS and 100 U/ml penicillin and 100 µg/ml streptomycin. All cells were cultured at 37°C with 5% CO_2.

Detection of HCV NS3 proteins by western blotting. BHK-21 cells were incubated with activated rSFV particles, rSFVeNS3/4A and rSFVe-sHELP-NS3/4A, with a multiplicity of infection of 10 for 24 hours at 37°C. The medium of the BHK-21 cells was removed and the cells were washed twice with PBS. Cells were lysed with cold TENT-SDS lysis buffer (150 mM NaCl, 50 mM Tris, 5 mM EDTA, 0.5% Triton-X-100, 1% SDS, pH7.5) containing 0.2 mM phenylmethanesulfonylfluoride (PMSF). Cell lysates were analyzed by 12% mini-protean TGX stain-free polyacrylamide gel (Bio-Rad, Veenendaal, The Netherlands) and western blotting. The tryptophan content was analyzed using Gel Doc EZ imager (Bio-Rad). HCV NS3 proteins were detected with goat anti-NS3 Ab at 1/1000 dilution (Abcam, Cambridge, UK). Alkaline phosphatase-conjugated rabbit anti-goat IgG Ab at 1/2000 dilution (Southern Biotech, Alabama, USA) was used as secondary antibodies. The NS3 proteins were visualized by colorimetric detection using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate substrates solution (Roche applied science, Almere, The Netherlands).

Mice and rSFV immunization. Female C57BL/6OLaHsd mice (8-10 weeks of age) were obtained from Harlan CPB, Zeist. Mice received intramuscular injections of rSFV (5×10^6 particles/mouse) on day 0 and day 14 of the experiment. All animal experiments were approved by the local Animal Experimentation Ethical committee (the Institutional Animal Care and Use Committee of the University Medical Center of Groningen).

Protein stability detected by [^{35}S]-methionine/cysteine pulse-chase labeling. BHK-21 cells (5×10^5 cells in a well of a 6-well plate) were incubated with 5×10^6 recombinant rSFVeNS3/4A or rSFVe-sHELP-NS3/4A particles. After 6 hours, supernatant was removed and the cells were washed with phosphate buffered saline (PBS). The cells were then further cultured in L-methionine and L-cysteine-free DMEM for 30 min following addition of [^{35}S]-

methionine/cysteine mix (0.37 Mbq/well) (PerkinElmer, Groningen, The Netherlands). After 1 hour of [^{35}S]-methionine/cysteine labeling, cells were washed with PBS and further cultured in medium supplemented with 5 mM L-methionine and L-cysteine. Cells were then washed and lysed with TENT-SDS lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, and 0.5% Triton-X-100, 1% SDS, pH7.5) containing 0.2 mM PMSF at 1, 6, 18, 24 and 42 hours after [^{35}S]-methionine labeling. Cell lysate was analyzed by SDS/PAGE and autoradiography.

Detection of HCV NS3-specific CD8$^+$ T cells. After removal of erythrocytes, peripheral blood cells and splenocytes were stained with HCV-NS3$_{603\text{-}611}$-PE dextramers (Immudex, Copenhagen, Denmark) for 10 minutes at room temperature. T cell phenotypes were characterized with the following antibodies: anti-CD8a-PE-Cy7 (53-6.7), anti-CD44-PerCp-Cy5.5 (IM7), anti-CD62L-APC (MEL-14) and anti-CD127-eFluor450 (A7R34). Surface staining was performed at 4 °C for 20 min. Antibodies were obtained from eBioscience (Vienna, Austria). Propidium Iodide (eBioscience) or DAPI were used as live/death cells distinction. Samples were analyzed using a LSR-II cytometer (BD Biosciences, Breda, The Netherlands). FACS data were analyzed using Flowjo software (Tree Star).

Proliferation assay. Splenocytes were labeled with 5 μM carboxyfluorescein diacetate succinimidylester (CFSE, Life Technologies) in PBS at 37°C for 10 min in dark. Labeled cells were washed twice with PBS cultured with medium only or with irradiated (100 Gray) Hepa1-6^V-nsPs cells at a ratio of 25:1 for 4 days at 37°C. After four days of incubation, the cells were harvested and stained with anti-CD8a-PE-Cy7 (53-6.7, ebioscience) at 4°C for 20 min. FACS analysis was performed using a LSR-II cytometer (BD Biosciences). Data were analyzed using Flowjo software (Tree Star).

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Identification and Validation of Hepatitis C Virus Cytotoxic T Lymphocyte Epitopes

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Identification and Validation of Hepatitis C Virus Cytotoxic T Lymphocyte Epitopes

ABSTRACT

Vaccine design requires prediction and validation of immunogenic MHC class I epitopes expressed by target cells as well as MHC class II epitopes expressed by antigen presenting cells essential for the induction of optimal immune responses. Epitope prediction methods are based on different algorithms and are instrumental for a first screen of possible epitopes. Yet their results do not reflect a one-to-one correlation with experimental data. We combined several *in silico* prediction methods to unravel the most promising hepatitis C virus (HCV) vaccine epitopes and validated these epitopes in *in vitro* and *in vivo* studies. Resolution of HCV infection involves induction of a robust and broad-spectrum T-cell response. Cytotoxic T lymphocyte (CTL) epitopes within the HCV nonstructural proteins (nsP) were identified and proteasomal cleavage sites and helper T cell (Th) epitopes at close proximity to these CTL epitopes were analyzed using multiple prediction algorithms. Here we show that the combination of the algorithms strongly increased the prediction accuracy as validated by *in vitro* and *in vivo* evaluation of the affinity and functions of the predicted CTL epitopes, respectively. This combined *in silico* analysis enhances the preciseness for identification of functional HCV-specific CTL epitopes. The design of vaccine based on the combined prediction of CTL, Th epitopes and proteasomal cleavage sites do not only apply to HCV, but also to other viral and non-viral antigens in which T-cell responses play a crucial role.

INTRODUCTION

Hepatitis C virus (HCV) is a positive-stranded RNA virus that primarily infects and persists in human hepatocytes. Clearance of HCV involves killing of virus-infected cells by cellular immunity, particularly by cytotoxic T lymphocytes (CTLs). However, 80% of HCV-infected patients are not able to clear the virus and HCV infection persists. Development of chronic HCV results in liver cirrhosis and 10 to 15% of chronically infected patients develop hepatocellular carcinoma in 10 to 15 years after the primary infection. During disease progression, viral mutants arise due to a combined result of a high mutation rate of the HCV genome and immune pressure[1,2].

In 2011 two antiviral HCV protease inhibitors, telaprevir and boceprevir, were approved for treatment for chronic HCV infection next to/combined with the standard treatment with pegylated interferon alpha and ribavirin. Although the sustained antiviral response improved by the use of these new drugs, two-thirds of chronically HCV-infected patients are still non-responders to all available treatments[3,4]. Antiviral treatments aim to eliminate HCV by inhibiting HCV replication but do not induce HCV-specific CTLs which has been shown to be crucial for clearance of HCV-infected cells[5]. Therefore, to effectively control HCV infection, immune-boosting intervention such as therapeutic vaccines, that aim to induce robust cellular immunity and to eliminate HCV-bearing cells, should be developed and used in combination with current HCV therapies.

Spontaneous resolution of HCV infection has been positively correlated to the activation of a wide T-cell repertoire recognizing multiple HCV epitopes[6,7]. CTL epitopes can be predicted by mathematics algorithms that compare the protein sequence of interest to a large database of known epitopes. However, data on the *in vivo* function of predicted CTL epitopes is limited. Some protective CTL epitopes have been identified in HCV-infected patients[8-10]. Yet, selection of epitopes to be included in a personalized vaccine is crucial and should take into account differences in virus genotypes and subtypes and the presence of escape viral mutants. Thus, in order to define high potential candidates for a therapeutic HCV vaccine, it is crucial to identify circulating virus sequences and their corresponding antigenic epitopes by high throughput methods.

In this study, we aimed to set up a procedure to define and subsequently verify protective HCV CTL epitopes and Th epitopes. We selected immunogenic epitopes within conserved HCV proteins based on the results from several mathematic algorithms available. Next, we measured the binding affinity of the predicted CTL peptides to MHC class I molecules *in vitro*. Furthermore, we determined the immunogenicity of the identified epitopes in an *in vivo* animal model.

RESULTS

Selection and characteristics of HCV synthetic long peptides that may contain CTL epitopes

The MHC class I epitope prediction algorithms SYFPEITHI[11], NetMHCpan 2.8 (ref. 12) and Immune Epitope Database and Analysis Resource (IEDB)[13] were used to predict CTL epitopes within HCV nonstructural proteins (nsPs) that bind H-2K^b/D^b. The first round of HCV CTL epitope selection was based on the predictions by the most commonly used online algorithm, SYFPEITHI (**Table 1**). Eighteen CTL epitopes that scored higher than 20 by SYFPEITHI prediction were selected. In addition, $NS5B_{1\text{-}16}$ (H-2^d)[14] and $NS5B_{46\text{-}63}$ (multiple human MHC alleles)[15], which are know binders for other MHC alleles, were selected as negative controls. As a result, twenty CTL epitopes were further analyzed with the NetMHCpan 2.8 and IEDB prediction algorithms. The threshold for strong binder is < 0.5 (% rank) in both NetMHCpan 2.8 and IEDB. Seven out of eighteen SYFPEITHI predicted CTL epitopes were predicted by either NetMHCpan 2.8 or IEDB (**Table 1**). Surprisingly, the negative controls, $NS5B_{1\text{-}16}$ and $NS5B_{46\text{-}63}$ were predicted as strong binders by both NetMHCpan 2.8 and IEDB.

To test the performance of these selected algorithms, the binding capacity of the HCV synthetic long peptides (SLPs) containing the selected CTL epitopes based on the prediction of the three algorithms (**Table 1**) were determined on MHC class I expressing cells, RMA-S cells (**Figure 1**). One concentration (10 μM) of SLPs was used in this MHC stabilization assay as to screen the possible H-2D^b and H-2K^b binders. HCV SLPs were classified as weak, intermediate and strong binders according to the ability to stabilize MHC class I molecules on the surface of RMA-S cells (depicted by the level of fluorescence index). Known CTL epitope peptides of ovalbumin ($OVA_{257\text{-}264}$) and human papillomavirus (HPV $E7_{49\text{-}57}$) were included as positive controls for H-2K^b and H-2D^b binding, respectively. Among 13 predicted HCV H-2D^b binders, predicted by at least one algorithm (solid and half-open circles), there was one strong binder ($NS3_{507\text{-}524}$), two intermediate binders ($NS3_{547\text{-}563}$ and $NS5B_{329\text{-}346}$) and three weak binders ($NS3_{72\text{-}87}$, $NS3_{323\text{-}340}$ and $NS3_{601\text{-}618}$). Three predicted H-2K^b binders (open circles) stabilized H-2D^b molecules and were considered as intermediate binders ($NS3_{214\text{-}228}$, $NS3_{383\text{-}400}$ and $NS3_{525\text{-}542}$) (**Figure 1a**). In ten SLPs that were predicted to be H-2K^b binders by at least one algorithm (open and half-open circles), one was classified as an intermediate binder ($NS5B_{152\text{-}169}$) and two were weak binders ($NS3_{214\text{-}228}$ and $NS5B_{1\text{-}16}$). Two predicted H-2D^b binders (solid circle) stabilized H-2K^b molecules were considered as weak binders ($NS3_{165\text{-}180}$ and $NS5B_{329\text{-}346}$) (**Figure 1b**).

Binding affinity of HCV short peptides to MHC class I molecules

In general, SLPs that contain a CTL epitope predicted by two or more algorithms

Protein/ position	Sequence (CTL epitopes are underlined)	MHC I peptides prediction						MHC I stabilization assay (**Figure 1**)		
		SYFPEITHI (**>20 strong binding**)		NetMHCpan 2,8 (**<0,5 strong binding**)		IEDB (**<0,5 strong binding**)		Peptide concentration = 10 μM[1,2]		selected for short peptides synthesis (v)
		H-2D^b	H-2K^b	H-2D^b	H-2K^b	H-2D^b	H-2K^b	H-2D^b	H-2K^b	
$NS3_{72-87}$	IQMYTNVDQDLVGWPA	**24**	10	0,8	3	2,05	2,25	**+**	-	
$NS3_{165-180}$	KAVDFIPVENLGTTMR	**30**	8	2	32	2,5	18,5	-	**+**	
$NS3_{214-228}$	VPAAYAAQGYKVLVL	0	**22**	10	15	24	6,8	**++**	**+**	
$NS3_{323-340}$	ATPPGSVTVSHPNIEEVA	**23**	9	**0,08**	8	**0,3**	9,45	**+**	**-**	v
$NS3_{383-400}$	ALGINAVAYYRGLDVSVI	0	**22**	32	1,5	19,1	1,15	**++**	-	
$NS3_{507-524}$	AETTVRLRAYMNTPGLPV	**22**	11	**0,1**	**0,25**	0,7	0,7	**+++**	**-**	v
$NS3_{525-542}$	CQDHLEFWEGVFTGLTHI	0	**21**	50	32	13,95	7,9	**++**	-	
$NS3_{547-563}$	LSQTKQSGENFPYLVAY	**28**	**22**	**0,3**	1,5	**0,4**	1,15	**++**	-	
$NS3_{601-618}$	RLGAVQNEVTLTHPITKY	**29**	12	**0,08**	32	**0,2**	12,95	**+**	**-**	v
$NS5A_{58-75}$	HCGAEITGHVKNGTMRIV	**24**	8	5	32	2,65	36,5	-	-	
$NS5A_{98-115}$	CTPLPAPNYKFALWRVSA	**20**	12	7	32	19	19	-	-	
$NS5A_{140-157}$	CPCQIPSPEFFTELDGVR	**21**	**22**	8	1,5	6,3	**0,5**	-	-	
$NS5A_{269-284}$	ITRVESENKVVILDSF	**24**	7	4	50	5,1	45,5	-	-	
$NS5B_{1-16}$	SMSYSWTGALVTPCAA	13	11	2	**0,03**	5,3	**0,2**	**+**	**+**	v
$NS5B_{46-63}$	CQRQKKVTFDRLQVLDSH	15	11	15	**0,05**	27	**0,25**	**-**	**-**	v
$NS5B_{152-169}$	GGRKPARLIVFPDLGVRV	15	**22**	32	15	22	12,35	**-**	**++**	v
$NS5B_{249-266}$	ARVAIKSLTERLYVGGPL	**22**	12	0,8	3	1,35	2,8	-	-	
$NS5B_{329-346}$	VQEDAASLRAFTEAMTRY	**20**	12	**0,4**	1,5	9,7	13,95	**++**	**+**	
$NS5B_{402-419}$	HTPVNSWLGNIIMFAPTL	**21**	8	7	8	12	2,7	-	-	
$NS5B_{423-439}$	MILMTHFFSVLIARDQL	13	**21**	8	**0,17**	9,1	**0,3**	**-**	**-**	v

[1]Fluorescence index of H-2D^b: 0,2-0,5 +; 0,5-1,5 ++; > 1,5 +++
[2]Fluorescence index of H-2K^b: 0,2-2 +; 2-8: ++; > 8 +++

Table 1. Selection of synthetic long peptides containing CTL epitopes from HCV nsPs by prediction algorithms. Sequences of the long synthetic peptides, for which MHC binding affinity was shown in figure 1, are shown. Strong binders are depicted in bold and high-lined in grey. The cut off score of SYFPEITHI is set at 20, high score indicates strong binder. The cut off score of NetMHCpan 2.8 and IEDB is set at 0.5 (% rank), low score indicates strong binder.

were able to bind and stabilize MHC class I molecules (**Table 1**). However, some SLPs that contain CTL epitopes predicted only by SYFPEITHI did not bind to MHC class I molecules. Since SLPs contain extra amino acid flanking the predicted CTL epitopes, binding affinity of SLPs to MHC class I molecules may be reduced[16,17]. Thus, binding capacity of the CTL epitopes was confirmed with short synthetic peptides that contain only the CTL epitope. Short synthetic peptides were selected based on one of the following criteria: (i) CTL epitope has to be predicted by at least 2 algorithms and/or classified as a weak, intermediate or strong binder from

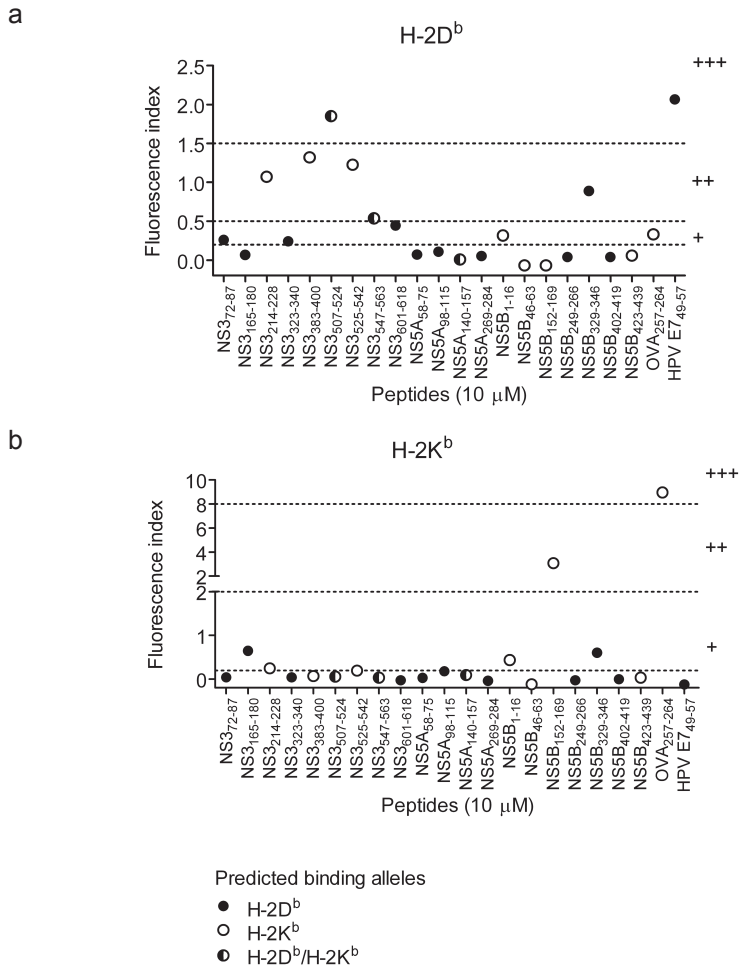

Figure 1. Stabilization of MHC class I molecules with binding of HCV SLPs. To induce MHC class I expression on cell surface, RMA-S cells were cultured at 26°C for 48 hours prior to the incubation with SLPs. Cells were then incubated with 10 μM of SLPs at 26°C for 4 hours, followed by a 1 hour cultured at 37°C. The expression level of surface MHC class I molecules, (**a**) H-2D^b and (**b**) H-2K^b, were analyzed using flow cytometry. HPV E7$_{49-57}$ and OVA$_{257-264}$ short peptides were positive controls for binding to H-2D^b and H-2K^b molecules, respectively. Dash lines indicate the cutoff values for H-2D^b (0.2-0.5: weak binders (+); 0.5-1.5: intermediate binders (++); > 1.5: strong binders (+++)) and H-2K^b (0.2-2: weak binders (+); 2-8: intermediate binders (++); > 8: strong binders (+++)).

the result of the MHC I stabilization assay with SLPs (NS3$_{323-340}$, NS3$_{507-524}$, NS3$_{601-618}$, NS5B$_{1-16}$, NS5B$_{46-63}$ and NS5B$_{423-439}$); (ii) CTL epitope has to be predicted by at least 1 algorithm and classified as intermediate or strong binder from the result of MHC I stabilization assay (NS5B$_{152-169}$). Since the accuracy of prediction greatly increased when peptides were analyzed with two or more algorithms, four extra CTL epitopes, which were predicted by at least two algorithms as strong binders, were selected (NS2$_{139-147}$, NS3$_{265-273}$, NS4B$_{38-46}$ and NS5A$_{280-287}$). And also for this analysis,

the known CTL epitopes of ovalbumin ($OVA_{257-264}$) and human papillomavirus (HPV $E7_{49-57}$), which are strong H-2K^b and H-2D^b binders, respectively, were selected as positive controls.

Binding affinity of selected short synthetic peptides on MHC class I molecules was determined by MHC class I stabilization assay with various concentrations of peptide (**Figure 2**). As expected, stabilization of both H-2D^b and H-2K^b molecules increased with increasing concentration of peptide. The kinetic of the stabilization of H-2D^b molecules was similar for all selected short peptides. The plateau of stabilization was reached at approximately 30 μM of peptides (**Figure 2a**). Two kinetic patterns of stabilizations of H-2K^b molecules were observed. For strong binders (at 100 μM, FI > 8; $OVA_{257-264}$, $NS5B_{2-10}$, $NS3_{265-273}$, $NS5B_{157-165}$, $NS2_{139-147}$ and $NS5B_{52-60}$), there was a dose dependent increase of FI and the plateau of stabilization was not detectable even at high peptide concentration (100 μM). For intermediate binders ($NS3_{514-522}$, $NS5B_{425-433}$ and $NS5A_{280-287}$), stabilization of H-2K^b molecules was modest at low peptide concentration (0.3–10 μM). However, the stabilization effect increased exponentially once the concentration was above 30 μM. This observation may explain the reason why we did not see strong H-2K^b binders when SLPs at 10 μM were used in the MHC I stabilization assay (**Figure 1**). Results of stabilization of MHC class I molecules with short peptides matched with the result from all of the MHC class I prediction algorithms used (H-2D^b: > 90% matched; H-2K^b: 100% matched) (**Table 2**).

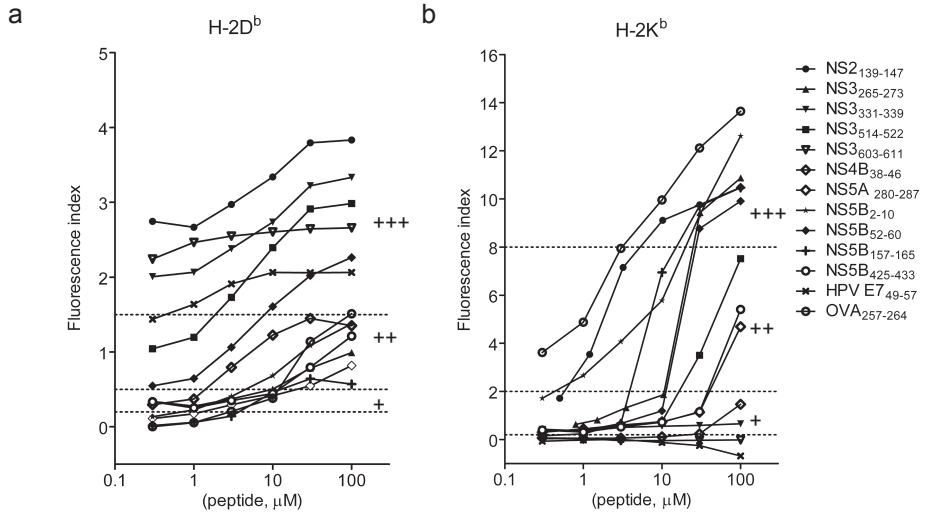

Figure 2. Binding affinity of HCV short peptides to MHC class I molecules. Short synthetic HCV peptides were serial diluted and incubated with RMA-S cells as described in figure 1. Dash lines indicate the cutoff values for H-2D^b (0.2-0.5: weak binders (+); 0.5-1.5: intermediate binders (++); > 1.5: strong binders (+++)) and H-2K^b (0.2-2: weak binders (+); 2-8: intermediate binders (++); > 8: strong binders (+++)).

The presence of proteasomal degradation sites at the carboxyterminal site of the predicted CTL epitopes

Under physiological conditions, proteins have to be cleaved by proteasomes into short peptides in order to be loaded on MHC class I molecules. Proteasomal cleavage sites were predicted with MAPPP, PAProC I and Netchop. MAPPP and Netchop predict the cleavage site of constitutive proteasomes and PAProC I predicts cleavage site of both constitutive proteasomes and immunoproteasomes. From the result of at least two prediction algorithms, proteasomal cleavage sites were present at all strong MHC class I binders (+++) (**Table 2**).

The presence of MHC class II epitopes flanking the predicted CTL epitopes

It has been observed that immunodominant CTL epitopes such as Influenza B nucleoprotein are flanked and/or overlap with Th epitopes[18-20]. Association of CTL and Th epitope may indeed enhance the immunogenicity of the CTL epitopes *in vivo*[21]. To investigate the presence of MHC class II epitope flanking the strong MHC class I binders, we next performed prediction of MHC class II epitopes with IEDB. Notably, most of the identified strong MHC class I strong binders were flanked with at least one MHC class II epitope. And three MHC class I strong binders (both H-2D^b and H-2K^b), overlap with an MHC class II epitope (IEDB rank < 10) ($NS2_{139-147}$, $NS3_{514-522}$ and $NS5B_{2-10}$). Flanking of MHC class I epitope with MHC class II epitope was also observed in our positive control, $OVA_{257-264}$ (**Table 2**).

Induction of peptide-specific effector CD8$^+$ T cells *in vivo*

Induction of *in vivo* T-cell response depends on both the presentation of peptides and the availability, avidity and affinity of precursor CD8$^+$ T cells[22,23]. Here, we investigated the induction of T-cell response against the predicted CTL epitopes in mice immunized three times with the rSFV particles expressing all HCV nsPs, NS3/4A or NS5A/B' (rSFVeNS2'-5B', rSFVeNS3/4A or rSFVeNS5A/B'). Splenocytes were isolated 1 to 3 weeks after the last immunization and re-stimulated with selected short peptides in order to induce degranulation (surface expression of CD107a/b) and secretion of IFN-γ by peptide-specific CD8$^+$ T cells (**Figure 3**). rSFV immunizations induced functional effector CD8$^+$ T cells (CD107a/b$^+$IFN-γ$^+$) against $NS2_{139-147}$, $NS3_{603-611}$, $NS5B_{2-10}$ and $NS5B_{157-165}$. When no peptides were added to the splenocytes, we already observed the presence of endogenous CD107a/b$^+$IFN-γ$^+$CD8$^+$ cells from mice immunized with any rSFV particles but not from mice immunized with PBS. This indicates that rSFV immunizations in general induced functional CD8$^+$ T-cell responses. In order to check the specific response against peptide, background (without peptide re-stimulation) subtraction was applied. Frequencies above zero indicate specific response against the re-stimulating peptide. CD8$^+$ T-cell response

a

Protein/position	Sequence	MHC I stabilization assay[1]	MHC class I prediction			Proteasomal cleavage			MHC class II prediction		
			SYFPEITHI (**>20 strong binding**)	NetMHCpan 2.8 (**<0,5 strong binding**)	IEDB (**<0,5 strong binding**)	MAPPP (cleavage probability[3])	PAProC I (Score[4])	Netchop (cleavage probability[3])	IEDB- H-2-IA^b (**<10 strong binding**)		
		H-2D^b	H-2D^b	H-2D^b	H-2D^b				5' of CTL epitope	complete CTL epitope	3' of CTL epitope
$NS2_{138-147}$	YVYNHLTPL	+++	13	**0,15**	0,7	1	0-	0,95	31,64 - 58,25	**1,82 - 3,90**	27,51 - 64,83
$NS3_{331-339}$	VSHPNIEEV	+++	**23**	**0,08**	**0,3**	0,7502	121+++	0,97	**2,21** - 12,13	10,72 - 48,25	31,02 - 54,21
$NS3_{614-622}$	RAYMNTPGL	+++	**22**	**0,1**	0,7	0	78++	0,95	**5,17** - 73,56	**5,97** - 22,88	16,57 - 87,59
$NS3_{630-638}$	GAVQNEVTL	+++	**29**	**0,08**	**0,2**	0	178+++	0,88	**9,35** - 55,70	18,51 - 32,13	16,70 - 51,34
HPV $E7_{49-57}$	RAHYNIVTF	+++	**23**	**0,08**	**0,2**	1	N.D.	0,91	33,38 - 53,38	56,31 - 79,26	69,56 - 86,81
$NS5B_{592-600}$	VTFDRLQVL	+++	15	15	27	0,5976	0-	0,96	10,39 - 86,63	71,97 - 79,66	28,42 - 85,75
$OVA_{257-264}$	SIINFEKL	++	0	4	**0,2**	1	N.D.	0,97	51,45 - 69,97	51,88 - 81,68	**9,57** - 82,66
$NS4B_{236-246}$	AVQTNWQKL	++	**23**	**1**	1,9	0	0-	0,72	12,81 - 32,62	23,98 - 74,51	21,59 - 58,54
$NS5B_{2-10}$	MSYSWTGAL	++	13	2	5,3	1	0-	0,87	N.D.	**0,93 - 0,95**	**2,62** - 56,47
$NS5B_{595-603}$	LMTHFFSVL	++	13	8	9,1	0,5009	40++	0,96	11,68 - 61,33	36,91 - 72,20	46,08 - 77,76
$NS3_{266-273}$	ITYSTYGKF	++	9	32	14,3	1	0-	0,209	10,30 - 47,29	36,17 - 47,83	49,48 - 84,06
$NS5A_{280-287}$	ILDSFDPL	++	0	15	**0,2**	0,906	0-	0,92	51,43 - 86,16	18,50 - 86,11	20,39 - 86,16
$NS5B_{157-165}$	ARLIVFPDL	++	15	32	22	0,6886	0-	0,96	44,16 - 67,37	47,47 - 56,37	51,99 - 81,87

b

Protein/position	Sequence	MHC I stabilization assay[2]	MHC class I prediction			Proteasomal cleavage			MHC class II prediction		
			SYFPEITHI (**>20 strong binding**)	NetMHCpan 2.8 (**<0,5 strong binding**)	IEDB (**<0,5 strong binding**)	MAPPP (cleavage probability[3])	PAProC I (Score[4])	Netchop (cleavage probability[3])	IEDB- H-2-IA^b (**<10 strong binding**)		
		H-2K^b	H-2K^b	H-2K^b	H-2K^b				5' of CTL epitope	complete CTL epitope	3' of CTL epitope
$OVA_{257-264}$	SIINFEKL	+++	**25**	1,5	**0,35**	1	N.D.	0,97	51,45 - 69,97	51,88 - 81,68	**9,57** - 82,66
$NS5B_{2-10}$	MSYSWTGAL	+++	11	**0,03**	**0,2**	1	0-	0,87	N.D.	**0,93 - 0,95**	**2,62** - 56,47
$NS3_{266-273}$	ITYSTYGKF	+++	11	**0,4**	**0,3**	1	0-	0,209	10,30 - 47,29	36,17 - 47,83	49,48 - 84,06
$NS5B_{157-165}$	ARLIVFPDL	+++	**22**	15	12,35	0,6886	0-	0,96	44,16 - 67,37	47,47 - 56,37	51,99 - 81,87
$NS2_{21-147}$	YVYNHLTPL	+++	11	**0,01**	**0,2**	1	0-	0,95	31,64 - 58,25	**1,82 - 3,90**	27,51 - 64,83
$NS5B_{592-600}$	VTFDRLQVL	+++	11	**0,05**	**0,25**	0,5976	0-	0,96	10,39 - 86,63	71,97 - 79,66	28,42 - 85,75
$NS3_{614-622}$	RAYMNTPGL	++	11	**0,25**	0,7	0	78++	0,95	**5,17** - 73,56	**5,97** - 22,88	16,57 - 87,59
$NS5B_{595-603}$	LMTHFFSVL	++	**21**	**0,17**	**0,3**	0,5009	40++	0,96	11,68 - 61,33	36,91 - 72,20	46,08 - 77,76
$NS5A_{280-287}$	ILDSFDPL	++	**21**	6	1,45	0,906	0-	0,92	51,43 - 86,16	18,50 - 86,11	20,39 - 86,16
$NS4B_{236-246}$	AVQTNWQKL	+	11	15	8,2	0	0-	0,72	12,81 - 32,62	23,98 - 74,51	21,59 - 58,54
$NS3_{331-339}$	VSHPNIEEV	+	9	8	9,45	0,7502	121+++	0,97	**2,21** - 12,13	10,72 - 48,25	31,02 - 54,21
$NS3_{630-638}$	GAVQNEVTL	-	12	32	12,95	0	178+++	0,88	**9,35** - 55,70	18,51 - 32,13	16,70 - 51,34
HPV $E7_{49-57}$	RAHYNIVTF	-	8	5	11,2	1	N.D.	0,91	33,38 - 53,38	56,31 - 79,26	69,56 - 86,81

[1]Fluorescence index of H-2D^b: 0,2-0,5 +; 0,5-1,5 ++; > 1,5 +++

[2]Fluorescence index of H-2K^b: 0,2-2 +; 2-8: ++, > 8 +++

[3]Cleavage probability: 1 = 100% cleavage

[4]PAProC I score: high score indicates high chance for cleavage, also depict by the number of +

Table 2. Detail analysis of selected short synthetic HCV peptides. Prediction of proteasomal cleavage sites and MHC class II epitopes flanking the selected CTL epitopes (**a**) H-2D^b, (**b**) H-2K^b. CTL epitope and its flanking amino acids (11 amino acid at 5' end and 11 amino acid at 3' end of the CTL epitope) were analyzed with MHC class II prediction algorithm (IEDB). Rankings below 10 were considered as strong binder. The values represent the range of ranking of the analyzed amino acids. Data were sorted according to the binding affinity of short peptides to RMA-S cells (**Figure 2**), strong binders at the top of the row (**a**: H-2D^b; **b**: H-2K^b). N.D. not determined.

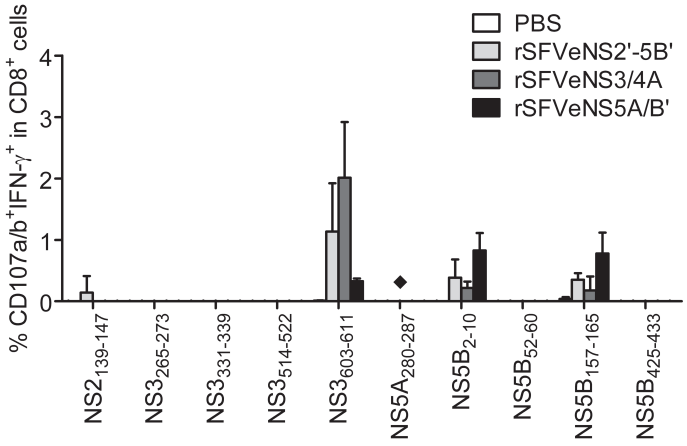

Figure 3. Induction of peptide-specific effector CD8⁺ T cells *in vivo*. Mice were intramuscularly immunized thrice with rSFV expressing HCV nsPs (rSFVeNS2'-5B', rSFVeNS3/4A or rSFVeNS5A/B') or PBS control with a one-week interval. Mice were sacrificed 1 to 3 weeks after the boost immunization. Splenocytes were isolated and cultured with 10 μg/ml of peptides for 5 hours before surface and intracellular staining. Background (splenocytes incubated with an equivalent concentration of DMSO) subtraction was applied and values above background are shown. Data represent combined results from three independent experiments, showing the mean +SEM (2-5 mice per group), PBS control (2 mice). ◆ one mouse per group.

6

against $NS3_{603-611}$ was the highest among all selected peptides and was detected in mice immunized with rSFVeNS2'-5B' or rSFVeNS3/4A. Responses against $NS5B_{2-10}$ and $NS5B_{157-165}$ were low and induced in mice immunized with rSFVeNS5A/B' or rSFVeNS2'-5B'. A very low response against $NS2_{139-147}$ was observed only in mice immunized with rSFVeNS2'-5B' but not in mice with other immunizations. Of note, all responding peptides were predicted by at least one algorithm for MHC class I prediction. Proteasomal cleavages site was presented in the carboxyterminal of all four peptides. Most strikingly, three peptides ($NS2_{139-147}$, $NS3_{603-611}$ and $NS5B_{2-10}$) contain MHC class II epitopes at close proximity of their CTL epitopes with relatively high ranking (**Table 2**).

DISCUSSION

Identification of protective HCV CTL epitopes is essential in the development of an efficient vaccine for patients with chronic HCV infection. Treatment can be personalized based on the circulating HCV genotype(s). Ideally one should identify HCV epitopes expressed by the patients' infected cells and induce T-cell responses against the identified epitopes with vaccine immunizations. Identification of HCV epitopes from cell lines expressing HLA-A2 and HCV proteins has been performed with electro-spray ionization quadrupole time of flight mass spectrometry/tandem mass spectrometry (ESI Q-TOF-MS/MS) and the data were consistent with the

results predicted from mathematic algorithms[24]. This method could also be used to identify naturally processed and presented HCV peptides from HCV-infected hepatocytes. However a widespread use of this analysis is limited as liver biopsies of individual patients are required for the analysis and taking biopsies is not standard procedure in these patients. Moreover, the percentage of HCV-infected hepatocytes in biopsies may be too low for MS analysis[25,26]. An alternative method is to identify CTL epitopes *in silico* based on the HLA phenotype and the viral sequence identified from blood of HCV-infected patients.

Here we show that the prediction accuracy for the identification of potential CTL epitopes is improved by combing several mathematic algorithms for MHC class I epitopes, MHC class II epitopes and proteasomal cleavage sites prediction. From all the HCV nsPs we narrowed down to 22 CTL epitopes by online prediction algorithms, of which 10 were proven to bind to H-2^b molecules on RMA-S cells. We next showed that immunization with rSFV expressing all nsPs induced a strong epitope-specific T-cell response against 1 out of 10 H-2^b binders and a weak response against 3 other epitopes.

Although the use of SLPs for the validation of the MHC class I binding capacity of CTL epitopes would allow a rapid screening of multiple epitopes within a protein, not all epitopes within such an SLP can bind to MHC molecules likely due to flanking amino acids. Thus only short peptides with the exact CTL epitopes can be used for this analysis.

Computational analyses used in this study are machine-learning algorithms, i.e., the programs are trained with large database for reliable result. Therefore, most algorithms favor the detection of immunodominant epitopes. Predictions are made with allele-specific motif methods that are based on amino acid sequence[27,28]. For example, peptide position 2 and 9 are classical anchor residues most important for binding to the HLA-A*0201 allele and are typically occupied by leucine, valine and isoleucine[29]. With allele-specific motif methods, peptides containing appropriate amino acid at anchoring positions are predicted as MHC class I binders but this type of prediction does not necessarily correlate with the physiological function of the epitopes.

Antigen processing and presentation involve a complex regulation of antigen degradation, interaction with chaperones, binding to the peptide-loading complex, loading onto MHC class I molecules and transport to the cell surface[30]. For both, presentation of intracellular antigens and cross-presentation of extracellular antigens, antigen degradation by constitutive and/or immune proteasome is required. Upon exposure of IFN-γ in lymphoid cells, the constitutive proteasomes are replaced by immunoproteasomes which have altered preference for cleavage site[31]. The immunoproteasomes favor the processing of immunodominant peptides[32]. Normal human liver cells also express immunoproteasomes besides constitutive and

intermediate proteasomes that enable the broadening of the repertoire of antigenic peptide[33]. In order to define the physiological function of predicted CTL epitopes, we analyzed the proteasomal cleavage site recognized by constitutive proteasomes (MAPPP, PAProC I and Netchop) or immunoproteasomes (PAProC I) present at the carboxyterminal of the predicted CTL epitopes. Combining MHC I prediction with proteasomal cleavage prediction indeed increases the accuracy of MHC I peptide prediction[34]. In this study, all predicted strong H-2D^b and H-2K^b binders contained proteasomal cleavage sites predicted by at least two algorithms.

Activation of CD8$^+$ T cells relies on both recognition of MHC I-peptide complex on APCs and co-stimulatory activation signals provided by adjacent cells such as APCs and/or CD4$^+$ helper T cells. Activated CD4$^+$ helper T cells orchestrate the activation of CD8$^+$ T cells by secretion of IL-2 and/or activation of dendritic cells for up-regulation of MHC I molecules and antigen presentation[21]. Interestingly, many known immunodominant CD8$^+$ T cell epitopes such as $OVA_{257-264}$ contain Th epitopes overlapping or at close proximity of one another[21,35]. This may allow presentation of both MHC I and MHC II peptides by the same dendritic cells resulting in activation of both CD8$^+$ and CD4$^+$ T cells simultaneously[36]. Another explanation is that activated helper T cells acquire MHC II-peptide complex from APCs as well as co-stimulating molecules leading to activation of Th1 responses and central memory CD8$^+$ T-cells responses[37-39]. It is proposed that the uptake the MHC II peptides may be further processed into MHC I peptides which can be loaded onto recycled MHC I molecules and present to CD8$^+$ T cells[21,40,41]. This will lead to higher activation of CD8$^+$ T cells as the helper T cells provide both antigen stimulation and co-stimulation[42]. Peptides recognized by splenocytes from rSFV-immunized mice ($NS2_{139-147}$ and $NS5B_{2-10}$) overlapped with strong Th epitopes (**Table 2**). The immunodominant epitope ($NS3_{603-611}$), which induces a very strong CD8$^+$ T-cell response in mice immunized with rSFVeNS2'-5B' or rSFVeNS3/4A, contains intermediate Th epitopes at the 5' ends of the CTL epitope. Remarkably, MHC class II epitope prediction by IEDB may not identify all possible Th epitopes, as for example, it has been shown that $E7_{44-62}$, binds to MHC class II molecules *in vitro* but it was not predicted as strong binders[43]. Beside from the results of prediction algorithm, the presence of Th epitope should be determined by *in vitro* experiments such as MHC class II binding assay.

In vivo, only 1 out of 22 predicted CTL epitopes induced strong CD8$^+$ T-cell responses and 3 out of 22 induced minor CD8$^+$ T-cell responses. Subdominant CTL epitopes could not be identified with rSFVeNS2'-5B', rSFVeNS3/4A or rSFVeNS5A/B' immunizations. Inclusion of more epitopes may increase competition between T cells of different specificity resulting in a reduced response to subdominant epitopes[44]. Furthermore, expression of full protein may reduce the expression level of one particular peptide compared to expression of only 1 peptide.

This may lead to insufficient presentation of antigen resulting in unstable contact between $CD8^+$ T cells and APC, thereby unable to fully activate T cells[45]. Although the biological function of responses against subdominant epitopes is unknown, an alternative approach to check the accuracy of *in silico* predictions is to immunize with synthetic long peptides expressing only one particular CTL epitope and its flanking sequences. In this way, immune response will be directed to that specific epitope. A major drawback of such an approach would be the number of mice that is required for this analysis.

In summary, we *in vivo* validated *in silico* approaches for epitope prediction. We showed that responses against predicted dominant CTL epitope were induced in mice immunized with HCV-nsPs expressing rSFV. Subdominant epitopes, which were predicted by the mathematic algorithms, may have to be confirmed with peptide immunizations. Furthermore, our studies confirm the observation that the presence of Th epitope in close proximity to CTL epitopes is a prediction for a dominant epitope. Combining with proteasomal cleavage sites and Th epitope algorithms increases the accuracy of CTL epitope identification. This will enable researchers to identify and narrow down the potential CTL epitopes as candidates for therapeutic vaccine development. For clinical practice, the establishment of an *ex vivo* stimulation assay with patients' PBMCs is warranted to select from the rationally designed candidate vaccines the one that is most immunogenic for that specific patient.

MATERIALS AND METHODS

Prediction algorithms and nucleotide sequences. HCV peptides associated with MHC class I molecules (H-2D^b and H-2K^b) were predicted with SYFPEITHI, NetMHCpan 2.8 and Immune epitope database and analysis resource (IEDB). HCV peptides associated with MHC class II molecules (H-2-IA^b) were predicted with IEDB. Proteasomal cleavage sites of the selected HCV peptides were predicted with MAPPP, PAProC I and Netchop. Protein analyzed: all nsPs of HCV genotype 1 (H77) (NCBI reference sequence: NC_004102.1), ovalbumin (GenBank: AAB59956.1) and E7 protein of Human papillomavirus type 16 (GenBank: AAL96657.1).

Synthetic peptides. Long synthetic peptides (13 to 18-mers) were kindly received from BEI Resources, NIAID, NIH: Peptide Array, Hepatitis C Virus, H77, NS2 protein, NR-3751; NS3 protein, NR-3752; NS4A protein, NR-3753; NS4B protein, NR-3754; NS5A protein, NR-3755; NS5B protein, NR-3756. Short synthetic peptides (8 to 9-mers) were manufactured by the department of Immunohematology, Leiden University Medical Center, The Netherlands. The purities of the synthetic peptide were analyzed with HPLC. All synthetic peptides have a purity of > 90%.

MHC class I stabilization assay. RMA-S cells were provided by Cornelis JM Melief (Leiden University Medical Center, The Netherlands) and were maintained in IMDM (Life Technologies, Bleiswijk, The Netherlands) supplemented with 10% FBS and 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies) at 37°C with 5% CO_2. RMA-S

cells were cultured with 5% CO_2 for 48 hours to induce expression of MHC class I. Cells were then incubated with various concentrations of synthetic peptides at 26°C for 4 hours, followed by cultured at 37°C for 1 hour. Cells were harvested, washed once with 0.5%BSA/PBS and stained with APC-anti-H-2K^b Ab (clone: AF6-88.5.5.3) and FITC-anti-H-2D^b Ab (clone: 28-14-8) (eBioscience, Vienna, Austria) at 4°C for 20 min. The surface expression of MHC class I molecules were analyzed by FACSCalibur cytometer (BD Bioscience) and data were analyzed with FlowJo software (Tree Star). Fluorescence index was calculated by dividing the median fluorescence intensity (MFI) value of cells incubated with SLPs by the MFI value of cells incubated with equivalent concentration of DMSO.

Mice. Specific pathogen-free female inbred C57BL/6JOlaHsd (H-2^b) mice were obtained from a commercial vendor (Harlan CPB, Zeist, The Netherlands) and were kept under the institute guidelines of the University of Groningen, The Netherlands. All mice were 8 to 10 weeks of age at the start of all experiments. Animal experiments were approved by the local Animal Experimentation Ethical Committee (the Institutional Animal Care and Use Committee of the University Medical Center of Groningen).

rSFV particles production and immunizations. rSFVeNS2'-5B', rSFVeNS3/4A and rSFVeNS5A/B' were produced as previously described[46]. Mice were primed and boosted immunized intramuscularly with a 2-week interval with 5×10^6 rSFV in 50 µl (25 µl/thigh muscle) under anesthesia (isoflurane/O_2). For negative controls, the same volume of PBS was injected intramuscularly.

Degranulation and IFN-γ staining. Splenocytes isolated from immunized mice were cultured with 10 µg/ml of synthetic peptides in the presence of anti-CD28 Ab (clone: PV-1, Bioceros B.V., Utrecht, The Netherlands), eFluor 660-anti-CD107a Ab (clone: eBio1D4B) and eFluor 660-anti-CD107b Ab (clone: eBioABL-93) in a 96-well plate at 37°C with 5% CO_2. One hour after culture, brefeldin A (1 mg/ml) was added and the cultures were further incubated for 4 hours. Cells were then harvested, washed and stained with LIVE/DEAD fixable violet dead cell stain kit (Life Technologies) according to manufacturer's manuals. Followed by surface staining with PE-Cy7-anti-CD8a Ab (clone: 53-6.7) at 4°C for 20 min and intracellular staining with PerCP-Cyanine5.5-anti-IFN-γ Ab (clone: XMG1.2) at 4°C for 30 min. All antibodies were purchased from eBioscience. FACS analysis was conducted with LSR-II flow cytometer, and data was analyzed with FlowJo software (Treestar).

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7

Summarizing Discussion and Future Perspectives

SUMMARIZING DISCUSSION

Our immune system is equipped with a natural ability to combat diseases such as infections and tumors. However, microbes and tumors have evolved mechanisms to escape from recognition by the immune system. In this way, microbes may persist and tumors may develop. To circumvent this phenomenon the immune system should be boosted. This can be achieved through the use of immunotherapy as a treatment regimen.

Hepatitis C virus (HCV) is one of the known human tumor-associated viruses that can evade the host immune system. More than 70% of the HCV-infected patients cannot clear the virus naturally and become chronic carriers. Persistent HCV infection results in chronic inflammation and thereby may lead to carcinogenesis as the worst possible outcome. Current standard treatment for chronic infection focuses on directly targeting the virus by using a combination of anti-viral drugs. Treatments targeting the immune system are warranted but still under development. In **Chapter 2**, we presented a summary of the recent literature on the development of immunotherapy against HCV infection. Most immunotherapeutic strategies have achieved potent anti-viral efficacy in preclinical studies. However, the translation of vaccine efficacy to HCV-infected patients has had limited success thus far. Despite the fact that most of the candidate vaccines were able to induce HCV-specific immune responses, they were not potent enough to resolve HCV infection. This indicates that there is room for improvement regarding vaccine efficacy.

The goal of the work described in this thesis was to develop an immunotherapeutic regimen against HCV infection. For this, we generated a therapeutic vaccine based on an alphavirus, Semliki Forest virus (SFV), to induce robust and long-lasting cellular immune responses.

Development of a therapeutic HCV vaccine based on SFV

Chronic HCV infection and HCV-induced hepatocellular carcinoma are often seen in combination with a poor and exhausted cellular immune response[1-3]. Thus, the capacity to induce a broad cellular immune response is an essential requirement for an effective therapeutic HCV vaccine. Others have shown that robust T-cell responses against the conserved nonstructural proteins (nsPs) of HCV are positively associated with spontaneous recovery from acute HCV infection[4-6]. In **Chapter 3**, we described the production and characterization of rSFV vectors encoding parts (NS3/4A or NS5A/B') or all nsPs (NS2'-5B') of HCV. Inclusion of all HCV nsPs (6.1 kb) increases the size of the recombinant RNA approximately 25% beyond the original size of the wild-type SFV RNA. And although this large genome reduces the production of rSFV 5-fold, the viral particles are functional. The particles express all HCV nsPs and *in vivo* induce protective nsPs-specific cellular immune responses. Inclusion of this large foreign gene in rSFV thus does not reduce the efficacy of the vaccine.

Compared to mice immunized with rSFV expressing HCV NS3/4A, mice immunized with rSFV expressing all HCV nsPs had lower numbers of $NS3_{603-611}$-specific $CD8^+$ T cells. Yet, this did not affect the anti-tumor activity. A reduced magnitude of the $NS3_{603-611}$-specific T-cell response may be due to differences in immunodominance hierarchy of the $NS3_{603-611}$ epitope induced by different rSFV immunizations (rSFVeNS3/4A versus rSFVeNS2'-5B'). Immunodominance hierarchy, the observation that an antigen-specific T-cell response of one specificity is stronger than the others with different specificities, is often associated with the frequency of antigen-specific precursor T cells and the abundance of antigen[7]. Reduced immunodominance hierarchy of $NS3_{603-611}$ after rSFVeNS2'-5B' immunization may be due to expression of other immunogenic epitopes (*e.g.*, $NS5B_{2-10}$ and $NS5B_{157-165}$) by this vaccine. A low magnitude of the $NS3_{603-611}$-specific T-cell response reduces the immune pressure on the virus and reduces the development of escape mutants[8-10]. Furthermore, immunization with rSFVeNS2'-5B' may allow induction of cellular immune responses against other CTL epitopes that are desirable for viral clearance[11,12].

Enhancement of SFV-based vaccine immunogenicity

In the studies described in **Chapter 4** and **5**, we tried to enhance the immunogenicity of the rSFV vaccine. This can be achieved with strategies such as an alternative immunization route[13], combination with immune modulators[14] or direct modification of the viral vector itself[15-17]. We directly modified the rSFV vaccine by inclusion of immunogenic carrier proteins that are commonly used to enhance immunogenicity of DNA vaccines[18]. Two carrier proteins, (i) tetanus toxin fragment C (TTFC) and (ii) a series of helper T cell (Th) epitopes fused with endoplasmic reticulum (ER) targeting signals (sigHELP-KDEL), were selected based on their high potency in DNA vaccines[19,20]. We studied the effect of these carrier proteins on rSFV expressing proteins of Human papillomavirus (HPV) (**Chapter 4**) or HCV (**Chapter 5**).

Inclusion of sigHELP-KDEL in an HPV rSFV-based vaccine led to enhanced immunogenicity, next to increased efficacy of eradication of TC-1 tumors in mice. Immunization with sigHELP-KDEL-expressing HPV rSFV vaccines resulted in doubling of the number of HPV E7-specific T cells from approximately 3% to 6% of the total $CD8^+$ T cell population compared to immunization with HPV rSFV vaccines without the inclusion of sigHELP-KEDL. The enhanced immunogenicity is attributed to the increased antigen production and stability by re-locating the antigen into the ER as well as by stimulation of $CD4^+$ T cell activation (**Chapter 4**). Enhanced immunogenicity was not observed when TTFC was incorporated in rSFV. TTFC is a relatively large protein that may contain CTL epitopes that could alter the immunodominance hierarchy of the antigen of interest and may not enhance the $CD8^+$ T-cell response against the $E7_{49-57}$ peptides as expected[21].

Interestingly, we observed that inclusion of sigHELP-KDEL into HCV-

expressing rSFV (rSFVeNS3/4A) did not improve the stability of the protein and the immunogenicity of the vaccine (**Chapter 5**). As observed by Oosterhuis, inclusion of sigHELP-KDEL was also not effective in enhancing the immunogenicity of DNA vaccines expressing hepatitis B virus core protein, human gp100 and plasmodium berghei circumsporozoite protein[22]. Immunization with rSFVeNS3/4A already induced as much as 12% of $NS3_{603-611}$-specific cells in the total $CD8^+$ T-cell population. The $NS3_{603-611}$ epitope is an immunodominant CTL epitope and flanked with Th epitopes. The localization of Th and CTL epitopes at close proximity has been shown to be positively associated with the immunogenicity of the CTL epitope[23,24]. Because of the strong immunogenicity of $NS3_{603-611}$, the inclusion of immunogenic carrier proteins may not be able to further enhance the immunogenicity of this particular epitope.

The vaccine immunogenicity in these two studies was purely based on the evaluation of the $CD8^+$ T-cell response against one immunodominant epitope, HPV $E7_{49-57}$ or HCV $NS3_{603-611}$. In order to further investigate the role of sigHELP-KDEL on the overall vaccine immunogenicity, studies on the $CD8^+$ T-cell response against other (subdominant) CTL epitopes will have to be performed.

Design of personalized therapeutic vaccines against HCV

Since chronic HCV infection is often associated with the development of viral escape mutants, induction of protective immunity against both wild-type virus and escape mutants is needed for development of an effective therapeutic HCV vaccine[25]. Immune response against the wild-type virus can be induced by an off-the-shelf vaccine that induces responses against conserved HCV proteins. Vaccines against escape mutants can only be designed when the sequences of the escape mutants or the peptides presented on the human leukocyte antigen (HLA) molecules of the infected cells are identified. Analysis of the HLA ligandome (immunopeptidome), i.e., all the peptides presented on the HLA molecules of the HCV-infected cells, by mass spectrometry is an ideal solution. However, complete analysis of HLA ligandome often involves extensive use of patient material, such as liver biopsies, that is generally not obtained from every patient with chronic HCV infection. Another way to identify protective epitopes presented by the circulating virions is to obtain the genome sequence of the virus and identify CTL epitope using mathematic predictions[26,27]. In **Chapter 6**, we identified (non-mutated) HCV CTL epitopes *in silico* with combinations of mathematic algorithms (SYFPEITHI, NetMHCpan 2.8 and IEDB). CTL epitopes presented by $H\text{-}2K^b$ and $H\text{-}2D^b$ were identified and validated *in vitro* and *in vivo*.

In vivo evaluation demonstrated that immunization of C57BL/6 mice with rSFV expressing HCV nsPs induces CTL responses against four predicted CTL epitopes ($NS2_{139-147}$, $NS3_{603-611}$, $NS5B_{2-10}$ and $NS5B_{157-165}$). All these four epitopes contain a proteasomal cleavage site. Moreover, $NS2_{139-147}$, $NS3_{603-611}$ and $NS5B_{2-10}$

contain at least one Th epitope at close proximity to the CTL epitope. As described earlier, the presence of a Th epitope enhances the immunogenicity of the nearby CTL epitope[23,24]. It has been proposed that both CTL and Th peptides can be presented by the same dendritic cells resulting in activation of both $CD8^+$ CTL and $CD4^+$ Th cells simultaneously[28]. Another hypothesis is that Th peptides could be degraded into shorter CTL peptide resulting in increased presentation of CD8 peptides[23]. In both scenarios, there is increased presentation of CTL epitopes and activation of $CD4^+$ Th cells which will eventually up-regulate the $CD8^+$ T-cell response.

Although responses against $NS2_{139\text{-}147}$, $NS5B_{2\text{-}10}$ and $NS5B_{157\text{-}165}$ were mild, possibly due to competition between epitopes[29], this result verified the accuracy of the mathematic predictions. To further confirm the *in vivo* functions of the predicted CTL peptides, immunizations with rSFV or other vaccine formats encoding the predicted CTL epitopes and their flanking Th epitopes will be required.

FUTURE PERSPECTIVES

Therapeutic HCV vaccine design

Antigen selection

Induction of a protective T-cell response against a broad-spectrum of viral epitopes is the main aim of a therapeutic HCV vaccine. A broad-spectrum of T-cell responses can be achieved by enclosing all conserved HCV proteins in a vaccine regimen. However, HCV evades our immune system and develops escape mutants[30]. Responses against epitopes derived from the escape mutant are also essential for clearance of virus-infected cells. Analysis of the HLA ligandome of the infected cells, which enables identification of all viral epitopes presented on the HLA molecules, would be the most precise way to select T-cell epitopes for a rational design of candidate vaccines.

Increasing antigen immunogenicity

When multiple antigens are incorporated in a vaccine, a broad immune response is induced. However, the magnitude of each T-cell response varies. This creates a vertical immunodominance hierarchy that may be different among different patients. Since the immunodominance hierarchy is determined by factors such as the availability of the antigen and the presence of a Th epitope[31], it is of crucial importance to maintain the "internal helps" in a vaccine especially in vaccine platforms that do not express the whole naïve antigen such as a peptide-based vaccine.

We propose that the immunodominancy hierarchy can be altered by inclusion of a potent carrier protein that provides "external help". As we have shown, the immunogenicity of HPV E7 was greatly enhanced by the inclusion of sigHELP-KDEL. Therefore, when "internal help" is not available in an antigen with low immunogenicity, a potent carrier protein with "external help" can be included in a vaccine for enhancement of immunogenicity.

Safety of a therapeutic HCV vaccine in humans

HCV normally infects less than ~10% of the total hepatocytes in humans[32]. The incidence of fulminant hepatitis or severe acute liver disease induced by HCV infection is rare and is only associated with high viremia[33,34] and a high percentage of infected hepatocytes (approx. 20%)[35]. Spontaneous recovery from acute HCV infection, which is associated with the presence of a strong and broad-spectrum T-cell response, does reduce viral titer without elevation of serum alanine aminotransferase (ALT), a marker for liver damage[36]. Even spontaneous recovery from chronic HCV infection, which is associated with increase neutralizing antibodies and restoration of T-cell function, does not lead to severe liver disease[37]. This implies that both the cytopathogenic damage by $CD8^+$ T cell and non-cytopathogenic clearance of virus by antiviral responses are responsible for the outcome of the disease.

Due to the limitation of our subcutaneous tumor mouse model, we could not evaluate the immuno-pathogenic effects on the liver. Association of HCV-specific $CD8^+$ T-cell response with liver damage was observed in a HCV transgenic mouse model expressing HCV structural protein in all hepatocytes[38]. Induction of cytotoxic effect by vaccinations is very unlikely due to the low infection rate of liver cell in most patients; however, special precautions could be needed when immunizing patients that have a very high frequency of infected liver cells or late stage liver disease.

Foreseeing future treatment for HCV-infected patients

The efficacy of HCV therapy recently improved because of the newly approved HCV-specific direct-acting antivirals (DAAs), telaprevir and boceprevir, in 2011 (ref. 39, 40). New DAAs, *e.g.*, simeprevir, sofosbuvir and faldaprevir will be approved in 2014-2015 (ref. 41). However, DAAs are costly and may induce side effects that result in early termination of therapy[42]. Furthermore, (memory) immune responses are not induced by DAAs, as they do not target to the immune system. Therefore immunotherapy either given as a single treatment modality or in combination with DAAs could further improve treatment success of patients with chronic HCV infections and may also prevent HCV reinfection when immune memory responses are induced. Curative treatment and prevention of reinfections would contribute greatly to the global control of HCV infection.

As recommended by the European Association for the study of liver, all patients with HCV-related liver disease should be treated no matter the stage of liver fibrosis[43]. As a matter of fact, the treatment effect of currently available drugs is associated with the stage of liver disease. The earlier the stage, the better the prognosis preventing progression of liver fibrosis[44]. It is also expected that immunotherapy against HCV should be most effective at early stage of the liver disease due to the low extent of immune suppression and exhaustion[1]. Therefore, we propose that immunotherapy should be given to all HCV-infected patients at any stage of the

liver disease including those who are naïve to standard treatment, partial or non-responder to standard treatment. Vaccination with rSFV expressing the conserved genome of HCV will induce a broad immune response against the conserved HCV proteins. If HCV infection persists with the emerging escape mutants, that are also resistant to the current DAAs[45-48], a personalized vaccine will have to be designed based on the prediction with bioinformatics algorithms or actual identification of T cell epitopes with HLA ligandome analysis. This personalized vaccine could come as peptide, a viral vector (*e.g.*, rSFV) or other vaccine formats and used as a booster immunization in order to eradicate the escape mutants. Furthermore, based on the immunogenicity of the antigen, "external help" could be included into rSFV for better vaccine immunogenicity.

By combining rSFV immunizations with DAAs and other immunotherapies such as the immune modulators anti-CTLA-4 and anti-PD-L1 antibodies, treatment efficacy can possibly be further enhanced. DAAs can reduce viral load, while immune modulators can restore the exhausted HCV-specific cell[49-51]. Of course, the most appropriate combination and its toxicity will have to be evaluated.

CONCLUSIONS

Taken together, preclinical studies presented in this thesis provide more insight in the development of immunotherapy against HCV infection. By using a potent alphavirus vector, a broad and robust cellular immune response is induced. Furthermore, we showed that rSFV can be modified to enhance the vaccine's immunogenicity. This modified rSFV thus enhances responses against antigens with low immunogenicity. These features make rSFV a widely applicable platform for future vaccines. Lastly, we demonstrated that the choice of antigens to be included in a candidate vaccine could be predicted by mathematic algorithms combined with *ex vivo* validation. A precise design of HCV vaccines will be crucial for effective eradication of HCV.

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NEDERLANDSE SAMENVATTING

Achtergrond en doel van het onderzoek

Ons afweersysteem voorkomt en bestrijdt infecties veroorzaakt door micro-organismen zoals virussen, bacteriën en parasieten. Daarnaast kunnen cellen van het afweersysteem, immuuncellen, ook ontspoorde lichaamseigen cellen of cellen die dreigen te ontsporen herkennen en doden. Deze ontsporende cellen kunnen zich ontwikkelen tot kankercellen.

Anderzijds hebben micro-organismen en tumoren mechanismen ontwikkeld om de afweer te omzeilen of te onderdrukken. Deze mechanismen worden ook wel immuunevasie en immuunsuppressie genoemd. De gevolgen hiervan zijn duidelijk, mensen lopen infecties op die soms chronisch worden en ongeveer een derde van de mensen krijgt kanker.

Infecties kunnen soms behandeld worden met antivirale middelen (virale infecties) of antibiotica (bacteriële infecties), afhankelijk van de veroorzaker van de infectie. Standaard behandelingen van kanker zijn gebaseerd op chirurgie, chemotherapie en/of radiotherapie. Deze behandelingen zijn niet altijd toereikend waardoor patiënten chronische infecties of een ongeneeslijke vorm van kanker ontwikkelen.

Een benadering die op het gebied van de behandeling van infecties en kanker steeds veelbelovender wordt is 'immuuntherapie'. Het doel van immuuntherapie is de afweer zo sterk te activeren dat geïnfecteerde cellen of kankercellen herkend en gedood kunnen worden. In het toonaangevende wetenschappelijke tijdschrift Science werd immuuntherapie tot dé '*wetenschappelijke doorbraak van het jaar 2013*' verkozen.

In dit proefschrift is onderzoek beschreven naar de ontwikkeling van immuuntherapie gericht tegen hepatitis C virus infecties.

Hepatitis C virussen (HCV) behoren tot de zogenaamde tumorvirussen. Infectie met een tumorvirus kan uiteindelijk leiden tot het ontstaan van kanker. HCV kan het immuunsysteem omzeilen waardoor 70 procent van de geïnfecteerde patiënten het virus niet kwijtraakt en de infectie chronisch wordt. Een chronische HCV infectie leidt tot leverontsteking, wat kan leiden tot levercirrose en in een deel van de patiënten tot leverkanker, hepatocellulair carcinoom.

Wereldwijd zijn ongeveer 150 miljoen mensen chronisch besmet met het virus en sterven er jaarlijks 350.000 mensen aan een HCV-gerelateerde leverziekte. In Nederland zijn ongeveer 60.000 mensen besmet met HCV, vaak nog zonder het te weten. De standaard behandeling van HCV infecties bestond in Nederland tot voor kort uit een combinatie van ribavirine (een antiviraal middel) en interferon. Deze behandeling is in een deel van de patiënten effectief maar heeft ook veel bijwerkingen. Recent zijn er twee nieuwe antivirale middelen tegen HCV toegelaten die nu bij sommige patiënten toegevoegd worden aan de standaard behandeling.

Er zijn echter nog geen vaccins beschikbaar die kunnen beschermen tegen HCV en ook effectieve immuuntherapie gericht tegen HCV-geïnfecteerde cellen is

nog niet beschikbaar.

Het doel van het onderzoek zoals beschreven in dit proefschrift is de ontwikkeling van immuuntherapie tegen HCV infectie. Hiervoor zijn recombinante vaccins gemaakt en onderzocht die afgeleid zijn van een knaagdiervirus, het Semliki Forest virus (SFV).

SFV kan zodanig veranderd worden dat het virus een cel maar één keer kan infecteren en geen nieuwe virusdeeltjes maakt. Daarnaast kan het virus zodanig veranderd worden dat het eiwitten naar keuze gaat produceren. Een cel die geïnfecteerd wordt met zo'n recombinant virus deeltje zal een grote hoeveelheid van dit eiwit aanmaken en vervolgens afsterven. Deze afstervende cel wordt daarna opgenomen door antigeen-presenterende cellen die het eiwit presenteren aan andere cellen van het immuunsysteem. Het immuunsysteem maakt daarop afweercellen die HCV geïnfecteerde cellen kunnen herkennen en doden, zogenaamde cytotoxische T cellen (CTL).

In het inleidende **hoofdstuk 1** van dit proefschrift wordt het kader van het promotieonderzoek omschreven. Naast een inleiding in de immunologie en immuuntherapie worden HCV en humaan papillomavirus (HPV) geïntroduceerd. HPV is ook een tumorvirus dat in hoofdstuk 4 als een doelwitvirus gebruikt is om te onderzoeken of kankervaccins versterkt kunnen worden.

In **hoofdstuk 2** is recente literatuur samengevat die onderzoek beschrijft naar de ontwikkeling van immuuntherapie van HCV infecties. Veel van deze onderzoeken laten zien dat de ontwikkelde immuuntherapieën in proefdieren antivirale effecten bewerkstelligen. Helaas hebben deze behandelingen in patiënten lang niet zulke goede resultaten. Er worden wel immuunresponsen opgewekt maar die zijn niet voldoende effectief om de infectie te klaren.

Therapeutisch HCV vaccin gebaseerd op Semliki Forest virus

Patiënten met een chronische HCV infectie en HCV-geïnduceerd hepatocellulair carcinoom hebben vaak een zwakke, uitgeputte afweer tegen het virus. Patiënten die het virus snel kunnen klaren hebben daarentegen een sterke, brede afweer tegen het virus, met name tegen de meer geconserveerde eiwitten van het virus, de niet structurele eiwitten (nsPs). Idealiter induceert een therapeutisch HCV vaccin dus een brede afweer tegen de nsPs van HCV.

In **hoofdstuk 3** beschrijven we de ontwikkeling en karakterisering van drie recombinante SFV vaccins die een deel van de nsPs van HCV (NS3/4A of NS5A/B') of alle nsPs van HCV (NS2'-5B) tot expressie brengen, respectievelijk rSFVeNS3/4A, rSFVeNS5A/B' en rSFVeNS2'-5B'.

De inbouw van het RNA wat codeert voor alle nsPs (NS2'-5B') in de SFV vector maakt het recombinante SFV RNA ongeveer 25 procent groter dan het oorspronkelijke SFV RNA. En hoewel de productie van dit recombinante virus vijf keer lager is dan de virussen die een deel van de nsPs tot expressie brengen zijn de

virus deeltjes functioneel en leiden tot een nsP-specifieke afweercellen.

rSFVeNS2'-5B' immunisatie in muizen leidt tot lagere aantallen specifieke CTLs tegen een epitoop van het NS3 eiwit ($NS3_{603-611}$) dan immunisatie met SFVeNS3/4A, toch is er geen verschil in antitumor activiteit tussen beide vaccins. De lagere frequentie aan afweer cellen met het 'grotere' vaccin kan te wijten zijn aan zogenaamde immuun dominantie hiërarchie. De verlaagde immuun dominante hiërarchie van $NS3_{603-611}$ na rSFVeNS2'-5B' immunisatie kan veroorzaakt worden door de expressie van andere immunogene epitopen met dit 'grotere' vaccin. Echter, een meer gespreide afweer tegen meerdere epitopen is belangrijk zoals boven beschreven en kan ook belangrijk zijn om 'escape mutanten' te voorkomen. Concluderend, de HCV vaccins die ontwikkeld zijn activeren afweercellen die op hun beurt kankercellen die deze HCV nsPs tot expressie brengen herkennen en doden.

Verhoging van de immunogeniciteit van vaccins gebaseerd op SFV

In **hoofdstuk 4** en **5** onderzoeken we methoden om de immunogeniciteit van rSFV vaccins verder te verhogen. Dit kan op verschillende manieren. Hier onderzochten we het effect van de inclusie van immunogene carriereiwitten in de virale vector. Deze carriereiwitten verhogen de immunogeniciteit van DNA vaccins. Of dit mechanisme ook effectief is in virale vector vaccins was niet bekend.

Twee carriereiwit-cassettes i) tetanus toxine fragment C (TTFC) en ii) een serie helper T cel (Th) epitopen gefuseerd aan een endoplasmatisch reticulum (ER) targeting signaal (sigHELP-KDEL) werden ingebouwd in SFV vaccins. In **hoofdstuk 4** wordt het effect van deze eiwitten in een bestaand rSFV vaccin gericht tegen HPV en in **hoofdstuk 5** in een van de rSFV vaccins gericht tegen HCV beschreven.

Inclusie van sigHELP-KDEL in het HPV vaccin verhoogt het aantal HPV-specifieke CTLs en de antitumor activiteit van het vaccin. De sterkere immuunrespons kan te wijten zijn aan de verhoogde eiwit productie door de vaccins, door de verhoogde stabiliteit van het eiwit en door activering van Th cellen. Inclusie van TTFC verhoogt de immunogeniciteit van het rSFV-HPV vaccin niet. TTFC is een relatief groot eiwit dat ook CTL epitopen kan bevatten waardoor de hiërarchie in immuundominantie van de eiwitten die tot expressie komen kan veranderen.

Interessant is dat, in tegenstelling tot het SFV-HPV vaccin, de inclusie van sigHELP-KDEL in het SFVeNS3/4A vaccin de stabiliteit van het eiwit en de immuunrespons niet verhoogt. Anderen hebben laten zien dat inclusie van sigHELP ook niet in alle DNA vaccins leidt tot een verhoging van de immunogeniciteit.

Wellicht is het ook niet mogelijk om de al hoge respons tegen het immuundominante CTL epitoop van NS3 te verhogen. Het $NS3_{603-611}$ CTL epitoop wordt geflankeerd door Th epitopen en er zijn aanwijzingen dat wanneer CTL en Th epitopen elkaar overlappen of vlak bij elkaar liggen dit gepaard gaat met een hoge immunogeniciteit van het CTL epitoop. Een immunogeniciteit die wellicht optimaal is en niet verder versterkt kan worden.

In beide studies is een enkel CTL epitoop onderzocht. Verder onderzoek zal moeten uitwijzen of de inclusie van deze carriereiwitten wellicht andere responsen kan versterken.

Ontwerpen van gepersonaliseerde therapeutische vaccins tegen HCV

Chronische HCV infectie gaat vaak gepaard met het ontstaan van virus 'escape-mutanten', daarom moet een therapeutisch vaccin niet alleen immuniteit opwekken tegen het wild-type virus maar ook tegen de escape-mutanten. Immunisatie tegen het wild-type virus zou idealiter kunnen met een 'off-the-shelf' vaccin. Afweer tegen de escape-mutanten kan alleen opgewekt worden door het vaccin zodanig te ontwerpen dat cellen geïnfecteerd met escape-mutanten ook herkend worden. Voor de ontwikkeling van zo'n gepersonaliseerd vaccin is de sequentie van de escape mutant nodig en moeten de epitopen die gepresenteerd worden door de patiënt-specifieke HLA moleculen van HCV geïnfecteerde cellen geïdentificeerd worden, i.e. de HLA ligandome (immunopeptidome). Deze analyse kan gedaan worden met massaspectometrie ware het niet dat hiervoor veel HCV-geïnfecteerde cellen van de patiënt nodig zijn. Een andere benadering is een theoretische benadering waarbij gebruik gemaakt wordt van mathematische voorspellingen. In **hoofdstuk 6** is deze theoretische methode toegepast op een aantal geïdentificeerde HCV CTL epitopen. Vervolgens zijn deze epitopen *in vivo* en *in vitro* geïdentificeerd en gevalideerd.

Immunisatie met SFVeNS2'-5B leidt tot responsen tegen 4 voorspelde epitopen. Alle vier de epitopen kunnen ook op basis van theoretische voorspelling van de proteosomale klieving ontstaan en drie van de vier epitopen lijken geflankeerd te worden door, of liggen dichtbij een Th epitoop. Hoewel de immuun responsen laag zijn lijkt het resultaat de mathematische voorspelling te bevestigen. Verder onderzoek zal moeten uitwijzen of op basis van deze mathematische voorspellingen patiënt-specifieke vaccins ontworpen kunnen worden.

Concluderend

Het onderzoek in dit proefschrift vergroot ons inzicht in de ontwikkeling en de mogelijkheden van immuuntherapie gericht tegen HCV infectie. De SFV vaccins die ontworpen zijn induceren een robuuste cellulaire immuunrespons. Verder laten we zien dat rSFV vaccins geoptimaliseerd kunnen worden waardoor responsen tegen antigenen met een lage intrinsieke activiteit verhogen. Deze eigenschappen maken dat rSFV een veelbelovend platform kan zijn voor nieuwe vaccins. Tot slot hebben we laten zien dat de keuze van het antigeen wat ingebouwd moet worden in een vaccin voorspeld kan worden door mathematische algoritmen. De ontwikkeling van een HCV vaccin voor het behandelen van HCV patiënten is van groot belang, de kennis verkregen en beschreven in dit proefschrift brengt dit weer een kleine stap dichterbij.

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Publications

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